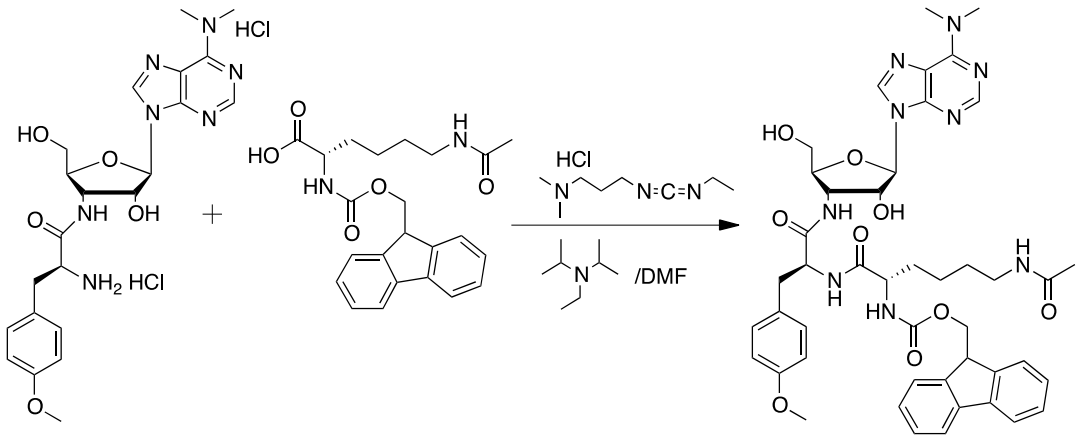
Supplementary Data

Synthesis of Fmoc-Lys(Ac)-Puromycin [α-Fmoc-Lys(ε-Ac)-puromycin].



Materials: puromycin dihydrochloride was purchased from Gold Biotechnology (CAS# 58-58-2, Catalog# P-600-1), α-Fmoc-Lys(ε-Ac)-OH was purchased from Novabiochem® (CAS# 159766-56-0, Catalog# 8.52042.0005, Lot# S6465442 249). And 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC•HCl) and 1-hydroxybenzotriazole (HOBt•H_2O) were purchased from Advanced Chem Tech. Dichloromethane (DCM) and dimethylformamide (DMF) were obtained from EMD Chemical. DMF was dried and purified by solvent pushstill (SG Water USA LLC, Nashua, NH). Ammonium formate was purchased from Fluka (HPLC, Catalog# 17843, Lot# BCBL7106V). Acetonitrile was purchased from Fisher Scientific (HPLC, Cat# A998-4). Purification was performed on Combiflash®R_f (Teledyne Isco) with RediSep®R_f High Performance GOLD 50 g HP C18 column (Teledyne Isco). ^{1}H NMR and ^{13}C NMR data were collected on 700 MHz Avance III Bruker and reported as chemical shift (δ) in ppm. Melting point (mp) was measured on Thomas Hoover Uni-melt capillary melting

point apparatus without calibration. Room temperature (rt) is 22 °C.

Synthesis of α-Fmoc-Lys(ε-Ac)-puromycin: To an oven-dried 20-mL screw-capped vial were added α-Fmoc-Lys(ε-Ac)-OH (226 mg, 0.55 mmol), EDC•HCl (105 mg, 0.55 mmol), HOBt•H_2O (74 mg, 0.55 mmol), and *N,N*-diisopropylethylamine (71.1 mg, 96 µL, 0.55 mmol) in 10 mL DMF. The mixture was stirred at rt for 1 h. Puromycin dihydrochloride (272 mg, 0.50 mmol, 1.0 eq) dissolved in 5 mL DMF was added *N,N*-diisopropylethylamine (129 mg, 174 µL, 1.0 mmol) drop-wise with stirring at 0 °C, then transferred to the α-Fmoc-Lys(ε-Ac)-OH DMF solution drop-wise at 0 °C. The reaction mixture was stirred at rt for 24 h. Upon the complete consumption of starting material, the reaction mixture was concentrated *in vacuo* to provide a light yellow paste. The crude mixture was loaded on Celite (3 g) with a minimum amount of water and acetonitrile. Purification of the eluate was performed with C18 column chromatography and two-step gradient elution (100% aqueous 100 mM ammonium formate, pH 6.6, to 70:30 formate buffer:acetontrile over 10 column volumes, then 40:60 formate buffer:acetonitrile over 15 column volumes). The fractions containing pure α-Fmoc-Lys(ε-Ac)-puromycin were combined, the acetonitrile was removed *in vacuo*, and the aqueous solution lyophilized. After lyophilization, α-Fmoc-Lys(ε-Ac)-puromycin was obtained as a white crystalline solid (190 mg, 44% yield). **mp**: 179.0–179.5 °C; **^{1}H NMR** (700 MHz, d_6-DMSO, 2.50 ppm) δ 8.46 (s, 1H), 8.23 (s, 1H), 8.15 (d, *J* = 6.9 Hz, 1H), 7.88 (m, 3H), 7.76 (b, 1H), 7.71 (dd, *J* = 7.5, 16.2 Hz, 2H), 7.50 (d, *J* = 8.1 Hz, 1H), 7.39 (dt, *J* = 7.4, 11.8 Hz, 2H), 7.32 (t, *J* = 7.3 Hz, 2H), 7.15 (d, *J* = 8.3 Hz, 2H), 6.79 (d, *J* = 8.4 Hz, 2H), 6.03 (s, broad, 1H), 6.01 (d, *J* = 1.0 Hz, 1H), 5.20 (t, *J* = 4.8 Hz, 1H), 4.60 (dd, *J* = 6.4, 12.1 Hz, 1H), 4.48 (m, 2H), 4.33-4.21 (m, 3H), 3.97 (d, *J* = 13.3 Hz, 1H),

3.91 (dd, *J* = 8.3, 13.3 Hz, 1H), 3.75-3.41 (m, 9H), 3.00-2.90 (m, 3H), 2.77 (dd, *J* = 8.9, 13.6 Hz, 1H), 1.76 (s, 3H), 1.55-1.40 (m, 2H), 1.38-1.28 (m, 2H), 1.26-1.08 (m, 2H); **^{13}C NMR** (176 MHz, d_6-DMSO, 40.45 ppm) δ 172.1, 171.8, 169.4, 158.3, 156.5, 154.8, 152.3, 150.1, 144.4, 144.2, 141.4, 139.9, 138.3, 130.8, 129.8, 128.1, 127.5, 127.4, 125.8, 121.8, 120.6, 120.1, 113.8, 110.2, 89.8, 83.6, 73.7, 66.2, 61.2, 55.0, 54.8, 54.3, 50.7, 47.1, 40.4 (underneath DMSO peak), 38.8, 37.7, 32.1, 29.3, 23.4, 23.0; **HRMS** (ESI-TOF, *m/z*): $[M+H]^+$ *calcd* $C_{45}H_{54}N_9O_9$ 864.4039; found 864.4039.

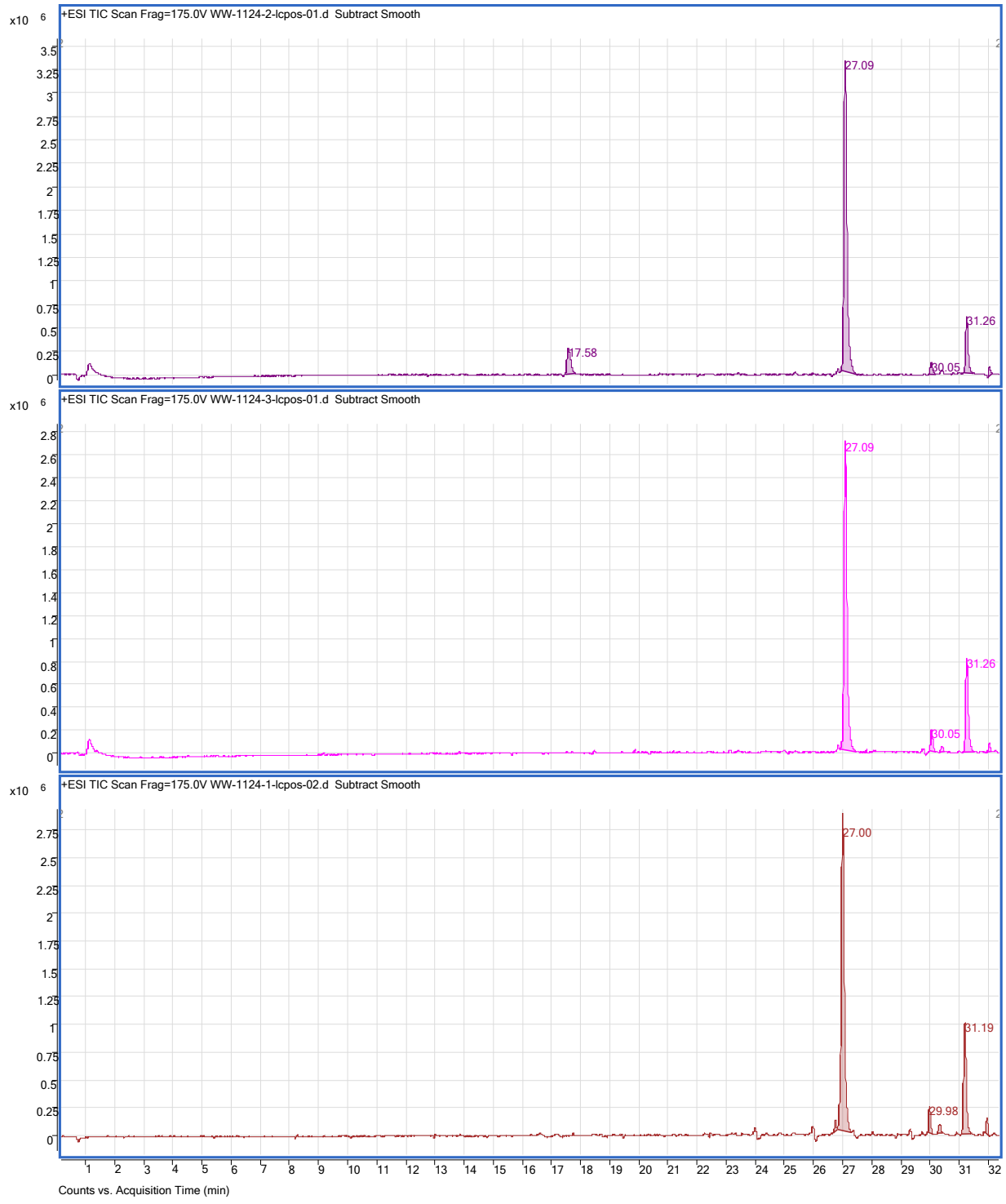
Verification of the stability and integrity of the prodrug.

The stability and the integrity of the DMSO stock solution of prodrug Fmoc-Lys(Ac)-Puromycin was verified by comparing with a standard Fmoc-Lys(Ac)-Puromycin retrieved from storage in a −20° freezer. Thin layer chromatography (TLC) was performed on a silica gel plate, developed with 3:7 acetonitrile: 100 mM ammonium formate (aq), and visualized under UV light (254 nm). The TLC result indicates that the DMSO solution remains identical to the standard Fmoc-Lys(Ac)-Puromycin with a similar purity.

Lanes from left to right: Sample 1, 20 mM stock solution of the prodrug Fmoc-Lys(Ac)-Puromycin in DMSO incubated at room temperature for 5 days; Co-spot, mixture of the Sample 1 and the standard Sample 2; Sample 2, the standard Fmoc-Lys(Ac)-Puromycin.

To further verify the integrity of the prodrug in DMSO stock solution, the samples were submitted for LCMS analysis. LCMS Sample 1 is the standard Fmoc-Lys(Ac)-Puromycin in methanol. LCMS Sample 2 is a methanol solution (2% DMSO) of the Fmoc-Lys(Ac)-Puromycin 20 mM in DMSO solution incubated at room temperature for 5 days. LCMS

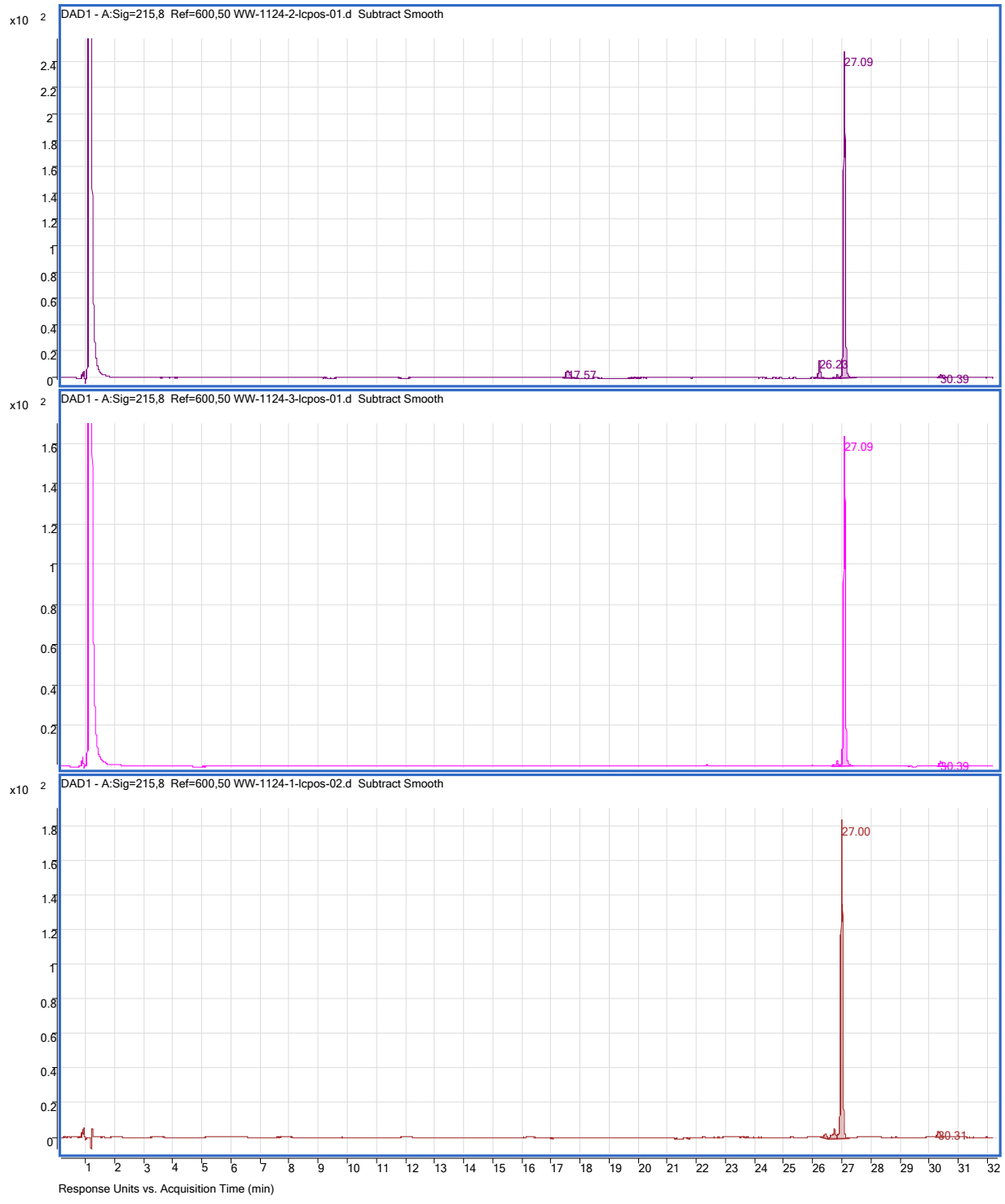
Sample 3 is the standard Fmoc-Lys(Ac)-Puromycin in methanol with DMSO (2%) added as control. The LCMS results indicate the standard Fmoc-Lys(Ac)-Puromycin is 93-96% pure (Sample 1 and Sample 3) based on their UV 215 nm integration, and the Sample 2 of the 20 mM DMSO solution of Fmoc-Lys(Ac)-Puromycin incubated at room temperature for 5 days is 88% pure based on UV 215 nm integration. The peak with a retention time (Rt) of 27 min corresponds to Fmoc-Lys(Ac)-Puromycin with an m/z value of 864.4. In Sample 2 there are two additional peaks. The first peak is at Rt 17.6 min and corresponds to Lys(Ac)-Puromycin ($[M+Na]^+$ m/z 664), 4.1 %), the second peak is at Rt ~26 min with m/z value 410.7 (6.7%) and is presumed to be a fulvene adduct. Overall the integrity of the 20 mM Fmoc-Lys(Ac)-Puromycin in DMSO solution incubated at room temperature for 5 days retained its integrity with approximately 4% degradation based on the percentage Lys(Ac)-Puromycin formed.



ESI+, TIC, m/z = 100-3200 mass chromatogram, Sample 2

ESI+, TIC, m/z = 100-3200 mass chromatogram, Sample 3

ESI+, TIC, m/z = 100-3200 mass chromatogram, Sample 1

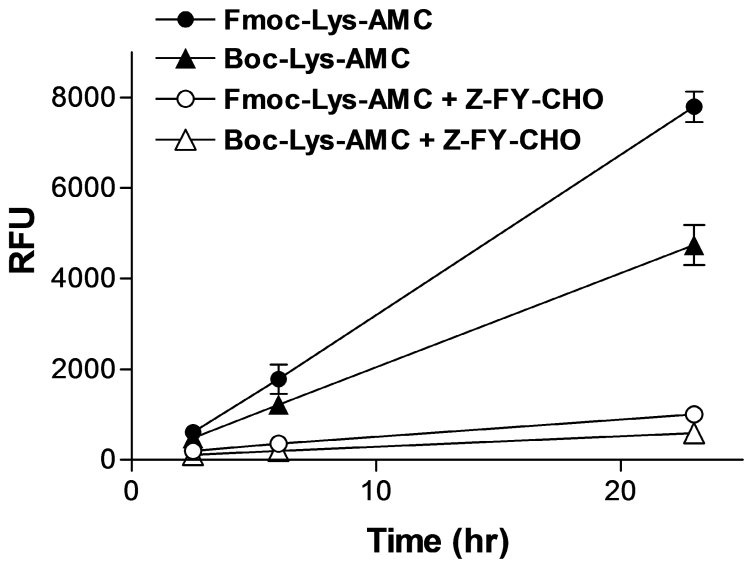


DAD1-A 215nm UV chromatogram, Sample 2

DAD1-A 215nm UV chromatogram, Sample 3

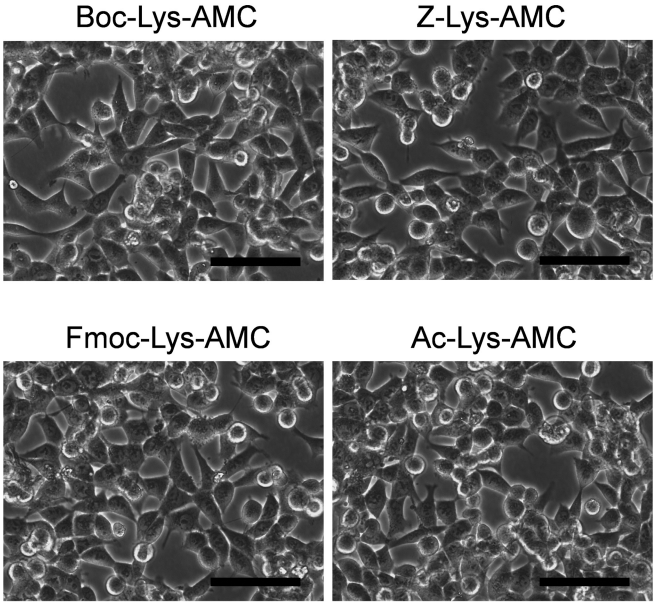
DAD1-A 215nm UV chromatogram, Sample 1

Supplementary Figure S1.



The reaction time period (20 h) in the live cell CTSL assay used in the Figure 2A was within a range of linear progression. The enzymatic assay were performed under the same condition as Figure 2A using HCT116 cells except for at different time points (2.5, 6 and 23 h).

Supplementary Figure S2.



Phase contrast images of cells to monitor morphological changes caused by cytotoxicity. HCT116 cells were incubated with either with Boc-Lys-AMC, Z-Lys-AMC, Fmoc-Lys-AMC or Ac-Lys-AMC (25 μM) for 20 h under the same experimental condition as Figure 2A in the main manuscript. Scale bar, 100 μm.

Supplementary Table S1. Complete blood count (CBC) after Fmoc-Lys(Ac)-Puromycin treatment.

CBC	No-treatment	Vehicle	5 mg/kg	15 mg/kg
WBC (10^3/μL)	7.3 ± 0.92	5.3 ± 0.61	5.5 ± 2.4	6.1 ± 1.6
RBC (10^6/μL)	10 ± 0.26	9.9 ± 0.61	9.2 ± 0.21	9.6 ± 0.058
Hemoglobin (g/dL)	17 ± 0.72	17 ± 0.55	15 ± 0.78	16 ± 0.31
Hematocrit (%)	55 ± 2.1	53 ± 1.7	51 ± 2.1	52 ± 0.58
MCV (fL)	53 ± 0.58	53 ± 2.1	55 ± 1.4	54 ± 0.58
MCH (pg)	16 ± 0.42	17 ± 1.0	17 ± 0.42	17 ± 0.35
MCHC (g/dL)	31 ± 0.67	32 ± 0.59	31 ± 0.28	32 ± 0.91
Platelet (10^3/μL)	670 ± 120	860 ± 290	1200 ± 2.1	1100 ± 260

Supplementary Table S2. Blood differential after Fmoc-Lys(Ac)-Puromycin treatment.

Differential	No-treatment	Vehicle	5 mg/kg	15 mg/kg
Neutrophils (/μL)	990 ± 340	850 ± 130	510 ± 190	750 ± 98
Bands (/μL)	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Lymphocytes (/μL)	5800 ± 560	4000 ± 550	5800 ± 2200	4900 ± 1600
Monocytes (/μL)	99 ± 47	120 ± 24	110 ± 48	75 ± 8.3
Eosinophils (/μL)	450 ± 180	350 ± 130	150 ± 5.7	340 ± 120
Basophils (/μL)	0 ± 0	0 ± 0	0 ± 0	0 ± 0