Supporting Information

Electrophilic PROTACs that degrade nuclear proteins by engaging DCAF16

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Methods

Common reagents and antibodies

The anti-HA (3724), FLAG (14793), DDB1 (6998), BRD4 (13440S), Lamin A/C (2032), K48-linked polyubiquitin (4289), HRP-linked rabbit IgG (7074) and HRP-linked mouse IgG (7076) antibodies were purchased from Cell Signaling Technology. The anti-FLAG HRP antibody (A8592), anti-FLAG affinity gel (A2220) and anti-HA agarose antibody (A2095) were purchased from Sigma-Aldrich. The anti-β-Actin antibody (sc-47778) was purchased from Santa Cruz Biotechnology. Alexa Fluor 488-conjugated goat anti-mouse IgG (H+L) secondary antibody (A-11001) and Alexa Fluor 568-conjugated goat anti-rabbit IgG (H+L) secondary antibody (A-11011) were purchased from Invitrogen. FuGene 6 (E2692) transfection reagent and sequencing grade modified trypsin (V5111) were purchased from Promega. Enzyme-linked chemiluminescence (ECL) (32106) and ECL plus (32132) western blotting detection reagents were purchased from Thermo Scientific. MG132 (S2619) was purchased from Selleck Chemicals. MLN4924 (15217), SLF (10007974) and JQ1 (11187) were purchased from Cayman Chemical. Polyethylenimine (PEI, MW 40,000, 24765-1) was purchased from Polysciences, Inc. Isotopically-labeled TEV-tags were synthesized as previously described[1].

Cell lines

Human Embryonic Kidney (HEK) 239T and MDA-MB-231 cells were obtained from ATCC and cultured in Dulbecco's Modified Eagle Medium (DMEM, Corning) with 10% (v/v) fetal bovine serum (FBS, Omega Scientific) and L-glutamine (2mM). For SILAC experiments, heavy and light labeled cell lines were cultured in DMEM medium with [$^{13}C_6$, $^{15}N_2$]-L-lysine and [$^{13}C_6$, $^{15}N_4$]-L-arginine (heavy labeled cells, 100 mg/mL each), or L-lysine and L-arginine (light labeled cells, 100 mg/mL each) for five generations. Both heavy and light SILAC medium were also supplemented with 10% (v/v) dialyzed FBS (Gemini), penicillin, streptomycin, and L-glutamine (2mM). Human Embryonic Kidney (HEK) 239 cells with DCAF16 gRNA CRISPR editing were purchased from Synthego and cultured in DMEM with 10% FBS and L-glutamine (2mM). All the cell lines were tested negative for mycoplasma contamination.

Cloning and mutagenesis

Human FKBP1A (FKBP12) cDNA with N-terminal FLAG tag or N-terminal FLAG tag and C-terminal nuclear localization sequence (NLS, PKKKRKV) was obtained by reverse transcription polymerase chain reaction (RT-PCR) amplification of a cDNA pool extracted from HEK293T cells and subcloned via EcoRI and BamHI sites into pCDH-CMV-MCS-EF1-Puro vector. Human DCAF16 cDNA with N-terminal HA tag was obtained by RT-PCR amplification of a cDNA pool extracted from HEK293T cells and subcloned via SalI and NotI sites into pRK5 vector. Human BRD4 with C-terminal FLAG tag was inserted into pRK5 vector by Gateway cloning technology. The expression vectors for DCAF16 mutants were generated by QuikChange site-directed mutagenesis.

Generation of FLAG-FKBP12 and FLAG-FKBP12_NLS stably expressed HEK293T cell lines by lentivirus transduction

FLAG-FKBP12 or FLAG-FKBP12_NLS lentivirus was generated by co-transfection of FLAG-FKBP12 or FLAG-FKBP12_NLS, pCMV-dR8.2 and pMD2.G into HEK 293T cells using FuGene 6 transfection reagent (Promega). Virus-containing medium were collected 48h after transfection, filtered with 0.45 μM filter, and used to transduce HEK293T and MDA-MB-231 cells in the presence of 10 μg/mL polybrene (Santa Cruz). 72h after transduction, puromycin (2 μg/mL) was added to cells. HEK293T cells stably expressing FLAG-FKBP12 or FLAG-FKBP12_NLS were obtained after puromycin selection for 7 days. HEK293T cells stably expressing pCDH empty vector were generated in parallel as control.

Generation of DTL and DCAF16 knockdown in HEK293T cell lines

shRNA lentivirus was generated by co-transfection of shRNA-containing vector, pCMV-dR8.2 and pMD2.G into HEK 293T cells using FuGene 6 transfection reagent (Promega). Virus-containing medium were collected 48h after transfection and used to transiently transduce HEK293T cells stably expressing FLAG-FKBP12_NLS for 48h. Lentiviral shRNAs targeting human DCAF16 or DTL were in pLKO.1 vector and purchased from Sigma. The sequences of shRNAs is described below.

shLuc (SHC007, Sigma):
CCGGCGCTGAGTACTTCGAAATGTCCTCGAGGACATTTCGAAGTACTCAGCGTTTTT

shDTL_1 (TRCN0000118815, Sigma):
CCGGCTGGTGAACTTAAACTTGTTACTCGAGTAACAAGTTTAAGTTCACCAGTTTTTG

shDTL_2 (TRCN0000118813, Sigma):
CCGGGCCTAGTAACAGTAACGAGTACTCGAGTACTCGTTACTGTTACTAGGCTTTTTG

shDCAF16_1 (TRCN0000122576, Sigma):
CCGGCAAACAGCTAAGCCGAACATTCTCGAGAATGTTCGGCTTAGCTGTTTGTTTTTTG

shDCAF16_2 (TRCN0000369937, Sigma):
CCGGTCCTGGTTGTATCATGCTAAACTCGAGTTTAGCATGATACAACCAGGATTTTTG

Generation of CRISPR-mediated knockout in HEK293 cell lines

DCAF16 CRISPR knockout HEK293 cell pools were generated by Synthego using nucleofection of Cas9-gRNA ribonucleoprotein (RNP) complex. Editing efficiency 48h post nucleofection of cell pool was 88%. HEK293 editing cell pools were subjected to single cell sorting in The Flow Cytometry Core Facility at Scripps Research. Individual cell clone was grown in 96-well plate until the cells were confluent. To confirm the editing in DCAF16 gene, genomic DNAs from each clone were extracted using PureLink Genomic DNA Mini Kit (Invitrogen). DCAF16 gene was amplified by PCR and confirmed by DNA sequencing. Indel analysis was performed using ICE analysis from Synthego (https://ice.synthego.com/#/). The sequences of DCAF16 gRNA and sequencing primers is described blow.

DCAF16 gRNA: TCTGACAAGTGGTCAGGAGA

DCAF16 sequencing primer (forward): TATTCAGGTATGGGAGTGGCTCTA

DCAF16 sequencing primer (reverse): GCCAGGATTTGAAGGAGATACTCT

To confirm DCAF16 knockout at the protein level, lentivirus containing FLAG-FKBP12_NLS was used to infect three wild type DCAF16 clones (clone 6, 17, 18) and three DCAF16 knockout clones (clone 3, 4, 22). Clone 6 stably expressing FLAG-FKBP12_NLS was grown in heavy SILAC DMEM medium for 5 generations. The other clones stably expressing FLAG-FKBP12_NLS were grown in light SILAC DMEM medium for 5 generations. All 6 clones were treated with 5 μM KB02-SLF and 10 μM MG132 for 2h. Cells were collected and analyzed for FLAG-FKBP12_NLS associating proteins using the same method described below. Proteins from clone 6 (heavy labeled) were combined with the other clones (light labeled) separately and identified by LC-MS/MS.

Cell lysis and Western blot

Cells were collected and lysed in 1% NP-40 lysis buffer (25 mM Tris-HCl pH 7.4, 150 mM NaCl, 10% glycerol, 1% Nonidet P-40) with cOmplete protease inhibitor cocktail (Roche). Cells were vortexed and sonicated for 5 pulses (40%, 4). The supernatant was collected after centrifugation at 16,000g for 10 min at 4 °C. Protein concentration was determined by DC assay (Bio-Rad). Protein lysate was heated at 95 °C for 5 min in 1X Laemmli sample buffer. Proteins were resolved by 12% or 14% Novex Tris-Glycine Mini Gels (Invitrogen) and transferred to polyvinylidene fluoride (PVDF) membrane (0.2 μM, Bio-Rad). The membrane was blocked with 5% BSA in TBST buffer (0.1% Tween 20, 20 mM Tris-HCl 7.6, 150 mM NaCl) at room temperature for 1h. The antibody was diluted with fresh 5% BSA in TBST buffer (1:10000 dilution for FLAG, HA and β-Actin, 1:1000 dilution for others) and incubated with membrane (1h room temperature for FLAG, HA and β-Actin, overnight 4 °C for others). Membrane was washed three times with TBST buffer and incubated with secondary antibody (1:5000 dilution in 5% BSA in TBST) at room temperature for 1h. Membrane was washed three times with TBST buffer. The chemiluminescence signal in membrane was recorded after developing in ECL or ECL plus western blotting detection reagent using CL-XPosure film (Thermo Scientific). Relative band intensities were quantified using ImageJ.

Subcellular fractionation

HEK293T cells stably expressing FLAG-FKBP12 or FLAG-FKBP12_NLS were harvested. Cell pellets were re-suspended in subcellular fraction buffer (250 mM Sucrose, 20 mM HEPES, pH 7.4, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, protease inhibitor cocktail) and homogenized on ice by 20 passes through a 25-gauge syringe needle. The pellets after centrifugation (720 g, 5min) were homogenized on ice by 10 passes through a 25-gauge syringe needle. Nuclear fraction was collected as the pellets after centrifugaing at 720 g for 5 min. The supernatant was ultracentrifuged at 100,000 g for 1 h. The resulting supernatant was the cytosol fraction. Equivalent portions of the cytosol and nuclear fractions were then subjected to Western blot analyses.

Immunoprecipitations

Cells were collected and lysed in 1% NP-40 lysis buffer with cOmplete protease inhibitor cocktail on ice for 10 min, followed by 5 pulses of sonication (40%, 4). After centrifugation at 16,000 g for

10 min at 4 °C, the supernatant was collected. FLAG affinity gel (20 µL slurry per sample) was incubated with protein lysates at 4 °C for 1h and washed four times with IP washing buffer. The affinity gel was heated at 95 °C for 10 min in 2X Laemmli sample buffer, followed by western blot analysis.

Immunofluorescence

Cells were seeded and grown in 35 mm glass bottom dish (MatTek). After compound treatment, cells were rinsed twice with PBS and fixed in 1 mL of 4% paraformaldehyde (v/v in PBS) for 15 min at room temperature. The fixed cells were rinsed twice with PBS, permeabilized and blocked with 0.1% Triton X-100 (v/v in 5% BSA in PBS) for 30 min at room temperature. The cells were incubated overnight (14h) at 4°C with FLAG or HA antibody at 1/100 dilution (in 0.1% Triton X-100/5% BSA in PBS). Cells were washed with 0.1% Triton X (in PBS) three times and incubated with Alexa Fluor 488-conjugated goat anti-mouse IgG (H+L) secondary antibody (for FLAG tag) or Alexa Fluor 568-conjugated goat anti-rabbit IgG (H+L) secondary antibody (for HA tag) at 1/1000 dilution (in 0.1% Triton X-100/5% BSA in PBS) at room temperature in dark for 1 h. Cells were washed with 0.1% Triton X (in PBS) three times and mounted with ProLong Gold Antifade Mountant with DAPI (invitrogen). Cells were imaged with Zeiss LSM780 in The Core Microscopy Facility at Scripps Research. Images were processed in ImageJ software. To quantify the degree of nucleus-localized fluorescence signal, background was subtracted, then the nuclear and whole cell area were selected and quantified for each cell examined. Relative nucleus with respect to whole cell fluorescence intensity was presented.

Identification of FLAG-FKBP12_NLS interacting proteins

HEK293T light and heavy SILAC cells that stably express FLAG-FKBP12_NLS were treated with DMSO and 5 µM KB02-SLF for 2h respectively, in the presence of 10 µM MG132. Heavy and light cells were collected and lysed in 1% NP-40 lysis buffer with cOmplete protease inhibitor cocktail. FLAG immunoprecipitation (20 µL slurry per sample) was performed with 2 mg of total protein lysates to enrich FLAG-FKBP12_NLS from light and heavy cell lysates. After washing the FLAG resin four times with IP washing buffer (0.2% NP-40, 25 mM Tris-HCl pH 7.4, 150 mM NaCl), FLAG resin from light and heavy samples were combined and washed once with PBS. FLAG-FKBP12_NLS and its associating proteins were eluted by heating at 65 ºC for 10 min with 8M urea in PBS, then reduced with 12.5 mM DTT at 65 ºC for 15 min and alkylated with 25 mM iodoacetamide at 37 ºC for 30 min. The protein solution was diluted with PBS to 2M urea and digested with 2 µg trypsin at 37 ºC for 6h. Tryptic peptides were acidified with 5% formic acid and loaded onto a silica capillary column (250 µm) packed with 3 cm of C18 resin (Aqua 5 µm, Phenomenex). Peptides were analyzed on LTQ-Orbitrap Elite mass spectrometer (Thermo Scientific) coupled with Thermo UltiMate 3000 UHPLC system. Peptides were separated on a capillary column packed with 3 cm of strong cation exchange (SCX) resin (Luna 5 µm, Phenomenex), 10 cm of C18 resin (Aqua 5 µm, Phenomenex) and a 5 µm tip. A five-step MudPIT method was used to analyze the peptides as previously described[2].

isoTOP-ABPP

HEK293T cells were treated with DMSO or 10 μM KB02-SLF for 2h. Cells were collected and subjected to isoTOP-ABPP sample preparation using the same protocol as previously described[1].

Proteome-wide identification of KB02-JQ1-induced protein degradation

HEK293T light and heavy SILAC cells were treated with DMSO and 5 μM of KB02-JQ1 for 24 h respectively. Light and heavy cells were collected and lysed in 1% NP-40 lysis buffer with cOmplete protease inhibitor cocktail. Cells were vortexed and sonicated for 5 pulses (40%, 4). The supernatant was collected after centrifugation at 16,000g for 10 min at 4 °C. Protein concentration was determined by DC assay. 50 μg proteome from light and heavy samples were mixed, followed by methanol/chloroform precipitation. Protein pellets were heated at 65 ºC for 10 min with 8M urea in PBS, then reduced with 12.5 mM DTT at 65 ºC for 15 min and alkylated with 25 mM iodoacetamide at 37 ºC for 30 min. The protein solution was diluted with PBS to 2M urea and digested with 2 μg trypsin at 37 ºC for 6h. Tryptic peptides were acidified with 5% formic acid. 5 μg peptides were loaded onto a silica capillary column (250 μm) packed with 3 cm of C18 resin. The same MudPIT method and CIMAGE software as described above were used to analyze the peptides.

Cell viability assay

HEK293T cells were seeded in 96-well clear bottom white plate (Corning) at 3×10^4 cells per well in 100 μL of DMEM medium and grown for 24h. The cells were treated with 0.625, 1.25, 2.5, 5, 10, 20 and 40 μM of KB02-SLF or KB02-JQ1 (DMSO stock, final DMEM concentration is 0.1% (v/v)) in 100 μL of DMEM medium for 24h. 50 μL of Cell Titer Glo reagent (Promega) was added to each well and incubate for 10 min at room temperature. The luminescence was read on CLARIOstar (BMG LABTECH).

qPCR analysis

Total RNA was extracted from the cells using RNeasy Mini Kit (Qiagen). cDNA was synthesized using iScript Reverse Transcription Supermix (Bio-Rad). Amplification of DCAF16, DTL and GAPDH genes was performed using SYBR Select Master Mix (Applied Biosystems) on an ABI Real Time PCR System (Applied Biosystems). The sequences of qPCR primers are described below. Relative DCAF16 and DTL gene expression was normalized to the GAPDH gene.

GAPDH primer (forward): CTGGGCTACACTGAGCACC

GAPDH primer (reverse): AAGTGGTCGTTGAGGGCAATG

DCAF16 primer (forward): AGTCTTGCCTGGCAGGTTAAG

DCAF16 primer (reverse): GGGACTTGTAAGAGGCTTTTGAA

DTL primer (forward): TCACTGGAATGCCGTCTTTGA

DTL primer (reverse): CTCACCAGCTTTTACGTCCC

LC-MS/MS detection of KB02-PEG0-SLF-modified tryptic peptides on DCAF16

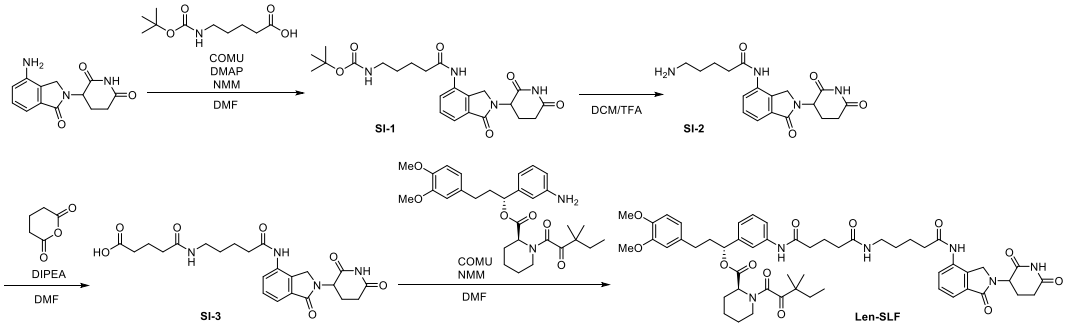
HEK293T cells were transfected with HA-DCAF16 plasmid by PEI transfection reagent for 24h and treated with DMSO or 10 μM KB02-PEG0-SLF for 2h. Cells were collected and lysed in 1% NP-40 lysis buffer with complete protease inhibitor cocktail. HA immunoprecipitation was performed with 10 mg of total protein lysates to purify HA-DCAF16. After washing the HA resin three times with IP washing buffer and once with PBS, HA-DCAF16 protein was eluted by heating at 65 ºC for 10 min with 8M urea in PBS, then reduced with 12.5 mM DTT at 65 ºC for 15 min and alkylated with 25 mM iodoacetamide at 37 ºC for 30 min. The protein solution was diluted with PBS to 2M urea and digested with 2 μg trypsin at 37 ºC for 6h. Tryptic peptides were acidified with 5% formic acid and loaded onto a silica capillary column (250 μm) packed with 3 cm of C18 resin (Aqua 5 μm, Phenomenex). Peptides were analyzed on LTQ-Orbitrap Elite mass spectrometer (Thermo Scientific) coupled with a Thermo UltiMate 3000 UHPLC system. Peptides were separated on a capillary column packed with 10 cm of C18 resin (Aqua 5 μm, Phenomenex) and a 5 μm tip. The flow rate was 0.5 μL/min. The gradient was 5% acetonitrile with 0.1% formic acid from 0-15 min, 5-30% acetonitrile with 0.1% formic acid from 15-149 min, 30-95% acetonitrile with 0.1% formic acid from 149-179min,95-5% acetonitrile with 0.1% formic acid from 179-180 min and 5% acetonitrile with 0.1% formic acid from 180-200 min. MS parameters were set as previously described[2]. The raw data was acquired in Xcalibur operation software.

Statistical analysis

Quantitative data were expressed in scatter plots with mean ± SEM (standard error of the mean, shown as error bar) shown. Differences between two groups were examined using unpaired two-tailed Student's t test. The P values were indicated (**P* < 0.05, ***P* < 0.01, ****P* < 0.001 and *****P* < 0.0001). P values < 0.05 were considered statistically significant.

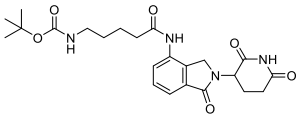
Compounds synthesis

Synthesis of lenalidomide-SLF



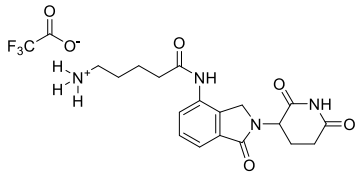
Scheme 1. Synthesis of Len-SLF

tert-butyl (5-((2-(2,6-dioxopiperidin-3-yl)-1-oxoisoindolin-4-yl)amino)-5-oxopentyl)carbamate (SI-1)



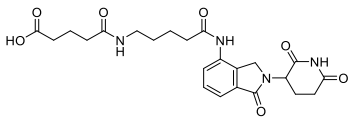
5-((tert-butoxycarbonyl)amino)pentanoic acid (823 mg, 0.37 mmol, 1 eq.), COMU (1.54 g, 0.36 mmol, 0.95 eq.), and N-methylmorpholine (840 µL, 7.6 mmol, 2 eq.) were dissolved in 4 mL of DMF and incubated for 1 min. Lenalidomide (648 mg, 2.5 mmol, 0.66 eq.) and catalytic 4-dimethylaminopyridine were added in 6 mL of DMF and the reaction was stirred at room temperature for 2h. The reaction mixture was diluted with ethyl acetate (20 mL), washed with 1N HCl (30 mL), water (30 mL), and brine (30 mL). The organic layer was dried over anhydrous $MgSO_4$ and filtered. This solution was left overnight at 4 ºC after which a white precipitate was found. The precipitate was filtered and dried to provide the title compound as a white amorphous solid (860 mg, 75%). ^{1}H NMR (600 MHz, DMSO-d6) δ 11.02 (s, 1H), 9.76 (s, 1H), 7.81 (dd, J = 7.4, 1.6 Hz, 1H), 7.54 – 7.40 (m, 2H), 6.81 (t, J = 5.7 Hz, 1H), 5.14 (dd, J = 13.3, 5.1 Hz, 1H), 4.38 (d, J = 17.4 Hz, 1H), 4.34 (d, J = 17.4 Hz, 1H), 2.97 – 2.89 (m, 3H), 2.61 (dt, J = 17.3, 3.3 Hz, 1H), 2.35 (t, J = 7.3 Hz, 3H), 2.03 (dtd, J = 12.5, 5.1, 2.2 Hz, 1H), 1.58 (p, J = 7.5 Hz, 2H), 1.42 (p, J = 7.2 Hz, 2H), 1.37 (s, 9H). HRMS (ESI) $[M+Na]^+$ for $C_{23}H_{30}N_4O_6Na$ 481.2057, found 481.2063.

5-amino-N-(2-(2,6-dioxopiperidin-3-yl)-1-oxoisoindolin-4-yl)pentanamide (SI-2)



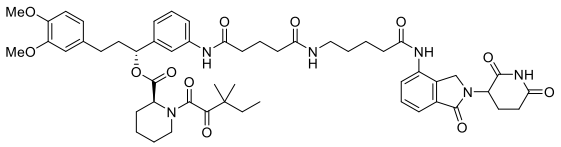
2 mL of dichloromethane, 2 mL of trifluoroacetic acid and 40 µL of H_2O was added to SI-1 (500 mg). The reaction was stirred at room temperature for 2h and monitored by TLC. The solvent was removed and the resulting powder was dried under vacuum overnight to provide the title compound as the TFA salt and used for the next step without purification.

5-((5-((2-(2,6-dioxopiperidin-3-yl)-1-oxoisoindolin-4-yl)amino)-5-oxopentyl)amino)-5-oxopentanoic acid (SI-3)



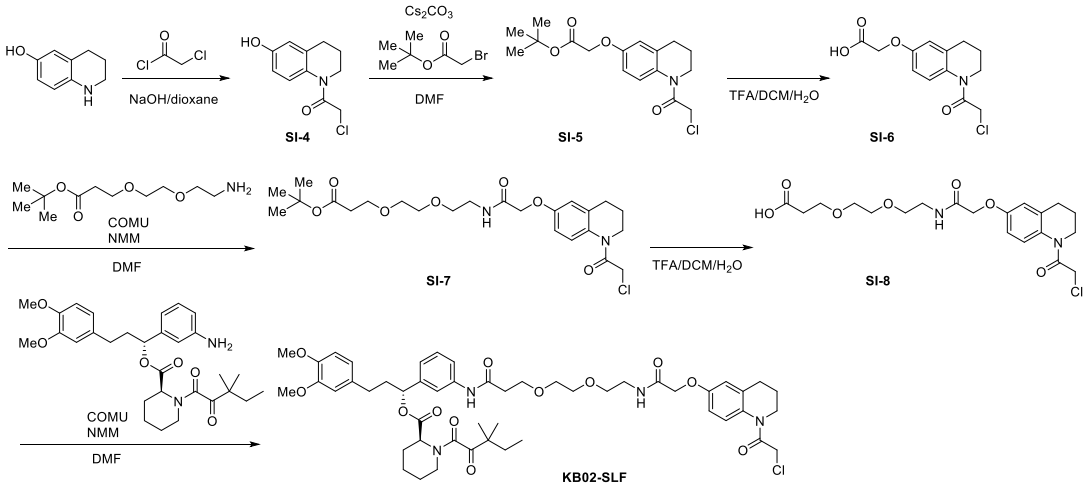
SI-2 (118 mg, 0.25 mmol, 1 eq.), glutaric anhydride (31 mg, 0.27 mmol, 1.1 eq.) and N,N-diisopropylethylamine (97 mg, 0.75 mmol, 3 eq.) were dissolved in 0.5 mL DMF and stirred at room temperature for 4h. Upon completion, the reaction was diluted to 1 mL with water/acetonitrile/formic acid (50/50/0.1) and purified by preparative HPLC to afford the title compound as a white powder after lyophilization (55 mg, 47%). ^{1}H NMR (600 MHz, Methanol-d4) δ 7.72 (d, J = 7.9 Hz, 1H), 7.65 (d, J = 7.5 Hz, 1H), 7.52 (t, J = 7.7 Hz, 1H), 5.16 (dd, J = 13.4, 5.2 Hz, 1H), 4.49 (d, J = 4.2 Hz, 2H), 3.22 (t, J = 7.0 Hz, 2H), 2.96 – 2.87 (m, 1H), 2.79 (ddd, J = 17.7, 4.6, 2.4 Hz, 1H), 2.55 – 2.43 (m, 3H), 2.32 (t, J = 7.4 Hz, 2H), 2.24 (t, J = 7.5 Hz, 2H), 2.19 (ddd, J = 10.6, 5.3, 2.7 Hz, 1H), 1.88 (p, J = 7.4 Hz, 2H), 1.74 (p, J = 7.5 Hz, 2H), 1.65 – 1.56 (m, 2H). HRMS (ESI) $[M+H]^+$ for $C_{23}H_{29}N_4O_7$ 473.2031, found 473.2038.

(1R)-3-(3,4-dimethoxyphenyl)-1-(3-(5-((5-((2-(2,6-dioxopiperidin-3-yl)-1-oxoisoindolin-4-yl)amino)-5-oxopentyl)amino)-5-oxopentanamido)phenyl)propyl (2S)-1-(3,3-dimethyl-2-oxopentanoyl)piperidine-2-carboxylate (Len-SLF)



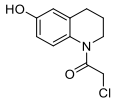
SI-3 (14 mg, 0.029 mmol, 1.5 eq.), COMU (12 mg, 0.028 mmol, 1.45 eq.), and N-methylmorpholine (6 µL, 0.057 mmol, 3 eq.) were dissolved in 150 µL of DMF and incubated for 1 min. SLF (10 mg, 0.019 mmol, 1 eq.) and catalytic 4-dimethylaminopyridine were added in 200 µL of DMF and the reaction was stirred at room temperature for 2h. The reaction was diluted to 1 mL with water/acetonitrile/formic acid (50/50/0.1) and purified by preparative HPLC to afford the title compound as a white powder after lyophilization (10.6 mg, 57%). ^{1}H NMR (600 MHz, DMSO-d_6) δ 11.02 (s, 1H), 9.93 (s, 1H), 9.79 (s, 1H), 7.82 (d, *J* = 1.4 Hz, 1H), 7.81 (d, *J* = 1.4 Hz, 1H), 7.70 (t, *J* = 1.9 Hz, 1H), 7.53 – 7.43 (m, 3H), 7.28 (t, *J* = 7.9 Hz, 1H), 7.01 (dt, *J* = 7.8, 1.3 Hz, 1H), 6.84 (d, *J* = 8.2 Hz, 1H), 6.76 (d, *J* = 2.0 Hz, 1H), 6.68 (dd, *J* = 8.2, 2.0 Hz, 1H), 5.63 (dd, *J* = 8.8, 4.8 Hz, 1H), 5.16 – 5.14 (m, 1H), 5.13 (d, *J* = 5.3 Hz, 1H), 4.38 (d, *J* = 17.4 Hz, 1H), 4.34 (d, *J* = 17.4 Hz, 1H), 3.72 (s, 3H), 3.71 (s, 3H), 3.31 – 3.28 (m, 1H), 3.16 (td, *J* = 13.3, 3.1 Hz, 1H), 3.07 (q, *J* = 6.9 Hz, 2H), 2.91 (ddd, *J* = 17.2, 13.6, 5.4 Hz, 1H), 2.60 (ddd, *J* = 17.5, 4.5, 2.3 Hz, 1H), 2.57 – 2.51 (m, 2H), 2.37 (qd, *J* = 8.0, 4.5 Hz, 3H), 2.29 (t, *J* = 7.5 Hz, 2H), 2.24 – 2.19 (m, 1H), 2.15 – 2.08 (m, 3H), 2.01 (dtd, *J* = 13.0, 5.3, 2.4 Hz, 2H), 1.79 (p, *J* = 7.5 Hz, 2H), 1.72 – 1.67 (m, 2H), 1.66 – 1.56 (m, 5H), 1.49 – 1.41 (m, 2H), 1.39 – 1.28 (m, 1H), 1.26 – 1.18 (m, 1H), 1.16 (s, 3H), 1.14 (s, 3H), 1.03 (d, *J* = 7.1 Hz, 1H), 0.80 (t, *J* = 7.5 Hz, 3H). HRMS (ESI) $[M+H]^+$ for $C_{53}H_{67}N_6O_{12}$ 979.4811, found 979.4808.

Synthesis of KB02-SLF



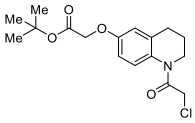
Scheme 2. Synthesis of KB02-SLF

2-chloro-1-(6-hydroxy-3,4-dihydroquinolin-1(2H)-yl)ethan-1-one (SI-4)



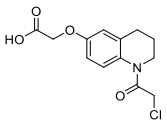
1,2,3,4-tetrahydroquinolin-6-ol (1 g, 6.7 mmol, 1 eq.) and NaOH (0.32 g, 8.0 mmol, 1.2 eq.) were dissolved in water/dioxane (1:1, 20 mL) at 0 ºC. Chloroacetyl chloride (0.59 mL, 7.4 mmol, 1.1 eq.) was added dropwise over 5 minutes and the reaction was stirred at room temperature for 4h. The reaction mixture was acidified with 1M HCl (pH < 4), extracted with ethyl acetate (2x30mL). The organic layers were combined and washed with brine (50 mL), dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure. The resulting residue was used for the next step without purification.

tert-butyl 2-((1-(2-chloroacetyl)-1,2,3,4-tetrahydroquinolin-6-yl)oxy)acetate (SI-5)



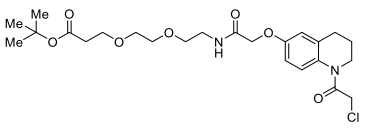
Cs_2CO_3 (3.2 g, 10.1 mmol, 1.5 eq.) and tert-butyl bromoacetate (1.3 mL, 8.4 mmol, 1.25 eq.) were added to a stirred solution of SI-4 (1.5 g, 6.7 mmol, 1 eq.) was dissolved in 15 mL DMF at room temperature. After 3h, the reaction was diluted with ethyl acetate (50mL), acidified with 1M HCl, then extracted with ethyl acetate (2x30 mL). The combined organic layers were dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The residue was used in the next step without further purification.

2-((1-(2-chloroacetyl)-1,2,3,4-tetrahydroquinolin-6-yl)oxy)acetic acid (SI-6)



SI-5 (981 mg, 2.9 mmol) was dissolved in 2 mL of dichloromethane, 2 mL of trifluoroacetic acid and 40 µL of H_2O. The reaction was stirred at room temperature for 2h. The solvent was removed under reduced pressure and the residue dried under vacuum to provide the title compounds as tan amorphous solid (623 mg, 76%) ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.33 (s, 1H), 6.82 – 6.65 (m, 2H), 4.65 (s, 2H), 4.55 – 4.35 (m, 2H), 3.68 – 3.64 (m, 2H), 2.67 (s, 2H), 1.88 (q, J = 7.1, 6.6 Hz, 2H). HRMS (ESI) $[M+H]^+$ for $C_{13}H_{15}ClNO_4$ 284.0684, found 284.0684.

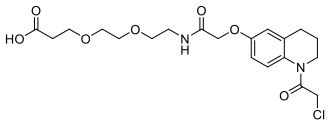
tert-butyl 3-(2-(2-(2-((1-(2-chloroacetyl)-1,2,3,4-tetrahydroquinolin-6-yl)oxy)acetamido)ethoxy)ethoxy)propanoate (SI-7)



SI-6 (100 mg, 0.35 mmol, 1.2 eq.), COMU (159 mg, 0.37 mmol, 1.2 eq.) and N-methylmorpholine (102 µL, 0.93 mmol, 3 eq.) were dissolved in 333 µL of DMF and incubated for 1 min. Amino-

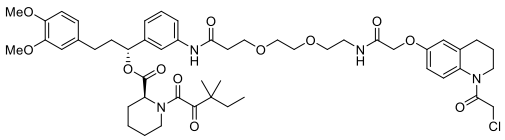
PEG2-t-butyl ester (90mg, 0.39 mmol, 1.1 eq.) was added in 667 µL and the reaction was stirred for 1h at room temperature. The reaction was diluted with ethyl acetate (10 mL) and water (10 mL). The organic layer was washed with water (3x10mL) and 1N HCl (1x10mL). The organic layer was collected, dried over anhydrous $MgSO_4$ and concentrated under reduced pressure. The residue was purified by flash chromatography (DCM:EtOAc; 1:1) to provide the title compound as a colorless oil (32 mg, 18%). ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.04 – 6.99 (m, 1H), 6.78 (dd, *J* = 8.8, 2.7 Hz, 1H), 6.76-6.71 (m, 1H), 4.48 (s, 2H), 4.19 (s, 1H), 4.05 (d, *J* = 1.0 Hz, 1H), 3.80 (s, 2H), 3.71 (dtd, *J* = 7.5, 6.4, 1.0 Hz, 2H), 3.63 – 3.57 (m, 6H), 3.56 – 3.53 (m, 1H), 3.50 (dd, *J* = 5.8, 4.8 Hz, 1H), 2.71 (s, 2H), 2.50 (qd, *J* = 6.3, 1.1 Hz, 2H), 2.01 – 1.95 (m, 2H), 1.44 (dd, *J* = 5.4, 1.1 Hz, 9H). HRMS (ESI) $[M+Na]^+$ for $C_{24}H_{35}ClN_2O_7Na$ 521.2025, found 521.2029.

3-(2-(2-(2-((1-(2-chloroacetyl)-1,2,3,4-tetrahydroquinolin-6-yl)oxy)acetamido)ethoxy)ethoxy)propanoic acid (SI-8)



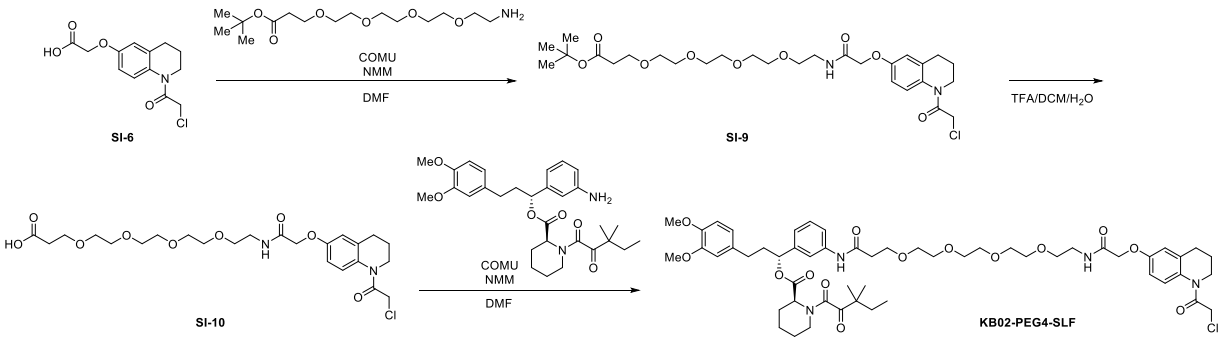
SI-7 (121 mg) was dissolved in 0.5 mL of dichloromethane, 0.5 mL of trifluoroacetic acid and 10 µL of H_2O and stirred room temperature for 2h. The solvent was removed under reduced pressure and the residue was dried under vacuum. The residue was used without further purification.

(R)-1-(3-(3-(2-(2-(2-((1-(2-chloroacetyl)-1,2,3,4-tetrahydroquinolin-6-yl)oxy)acetamido)ethoxy)ethoxy)propanamido)phenyl)-3-(3,4-dimethoxyphenyl)propyl (S)-1-(3,3-dimethyl-2-oxopentanoyl)piperidine-2-carboxylate (KB02-SLF)



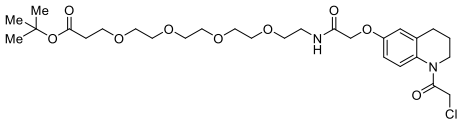
SI-8 (13 mg, 0.029 mmol, 1.5 eq.), COMU (12 mg, 0.028 mmol, 1.45 eq.), and N-methylmorpholine (6 µL, 0.057 mmol, 3 eq.) were dissolved in 150 µL of DMF and incubated for 1 min. SLF (10 mg, 0.019 mmol, 1 eq.) and catalytic 4-dimethylaminopyridine were dissolved in 200 µL of DMF and added to the reaction. The reaction was stirred at room temperature for 2h. The reaction was diluted to 1 mL with water/acetonitrile/formic acid (50/50/0.1) and purified via preparative HPLC and lyophilized to provide the title compound as a white powder (4.8 mg, 26%). ^{1}H NMR (600 MHz, DMSO-*d*6) δ 9.98 (s, 1H), 8.04 (t, *J* = 5.7 Hz, 1H), 7.70 (t, *J* = 1.9 Hz, 1H), 7.46 (ddd, *J* = 8.2, 2.1, 1.0 Hz, 1H), 7.29 (t, *J* = 7.9 Hz, 1H), 7.02 (dt, *J* = 7.8, 1.3 Hz, 1H), 6.84 (d, *J* = 8.1 Hz, 1H), 6.80 (o, 1H), 6.76 (m, 3H), 6.68 (dd, *J* = 8.2, 2.0 Hz, 1H), 5.63 (dd, *J* = 8.7, 4.8 Hz, 1H), 5.14 (d, *J* = 5.8 Hz, 1H), 4.44 (s, 2H), 3.72 (s, 3H), 3.71 (s, 3H), 3.68 (t, *J* = 6.3 Hz, 2H), 3.65 (t, *J* = 6.4 Hz, 2H), 3.49 (m, 4H), 3.43 (t, *J* = 6.0 Hz, 2H), 3.27 (q, *J* = 12.0 Hz, 2H), 3.15 (td, *J* = 13.2, 3.0 Hz, 1H), 2.66 (s, 2H), 2.54 (m, 4H), 2.53 (o, 2H), 2.22 (d, *J* = 13.5 Hz, 1H), 2.12 (br.s, 1H), 2.01 (m, 1H), 1.86 (br.s, 2H), 1.76-1.48 (m, 5H), 1.34 (m, 1H), 1.22 (m, 1H), 1.16 (s, 3H), 1.14 (s, 3H), 1.03 (d, *J* = 6.0 Hz, 1H), 0. 80 (t, *J* = 7.5 Hz, 3H). HRMS (ESI) $[M+H]^+$ for $C_{50}H_{66}ClN_4O_{12}$ 949.4360, found 949.4352.

Synthesis of KB02-PEG4-SLF



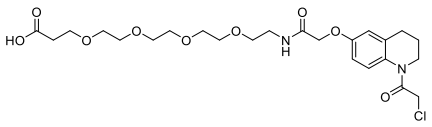
Scheme 3. Synthesis of KB02-PEG4-SLF

tert-butyl 1-((1-(2-chloroacetyl)-1,2,3,4-tetrahydroquinolin-6-yl)oxy)-2-oxo-6,9,12,15-tetraoxa-3-azaoctadecan-18-oate (SI-9)



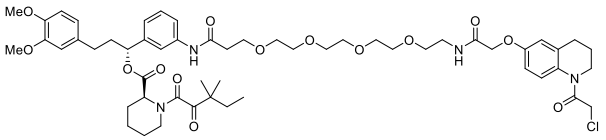
SI-6 (76 mg, 0.27 mmol, 1.2 eq.), COMU (115 mg, 0.27 mmol, 1.2 eq.), and N-methylmorpholine (74 µL, 0.67 mmol, 3 eq.) were dissolved in 333 µL of DMF and incubated for 1 min. Amino-PEG4-t-butyl ester (72 mg, 0.22 mmol, 1 eq.) was added in 667 µL and the reaction was stirred for 1h at room temperature. The reaction was then diluted with ethyl acetate (10 mL) and water (10 mL). The organic layer was washed with water (3x10mL) and 1N HCl (1x10mL). The organic layer was collected, dried over anhydrous $MgSO_4$ and removed under reduced pressure. The residue was purified by flash chromatography (DCM:EtOAc, 1:1) to provide the title compound as a colorless oil (44 mg, 22%). ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.10 (s, 1H), 6.81 – 6.69 (m, 2H), 4.46 (d, *J* = 11.3 Hz, 2H), 4.18 (s, 1H), 3.83 – 3.72 (m, 2H), 3.70 – 3.67 (m, 4H), 3.63 (s, 8H), 3.61 – 3.57 (m, 6H), 3.54 (dd, *J* = 7.6, 3.2 Hz, 2H), 2.70 (s, 2H), 2.48 (td, *J* = 6.5, 1.5 Hz, 2H), 1.98 (d, *J* = 5.2 Hz, 2H), 1.43 (d, *J* = 3.1 Hz, 9H). HRMS (ESI) $[M+H]^+$ for $C_{28}H_{44}ClN_2O_9$ 587.2730, found 587.2721.

1-((1-(2-chloroacetyl)-1,2,3,4-tetrahydroquinolin-6-yl)oxy)-2-oxo-6,9,12,15-tetraoxa-3-azaoctadecan-18-oic acid (SI-10)



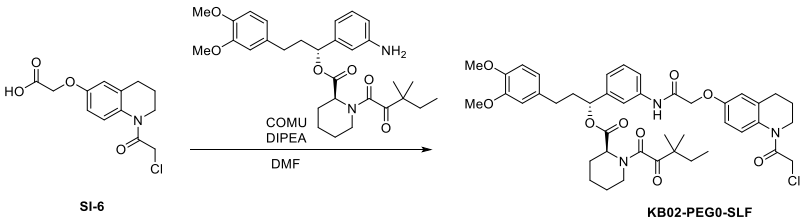
SI-9 (119 mg) was dissolved in 0.5 mL of dichloromethane, 0.5 mL of trifluoroacetic acid and 10 µL of H_2O and stirred at room temperature for 2h. Upon consumption of the starting material, the reaction was concentrated under reduced pressure and dried under vacuum. The resulting residue was used without further purification.

(R)-1-(3-(1-((1-(2-chloroacetyl)-1,2,3,4-tetrahydroquinolin-6-yl)oxy)-2-oxo-6,9,12,15-tetraoxa-3-azaoctadecan-18-amido)phenyl)-3-(3,4-dimethoxyphenyl)propyl (S)-1-(3,3-dimethyl-2-oxopentanoyl)piperidine-2-carboxylate (KB02-PEG4-SLF)



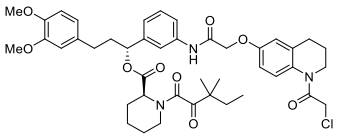
SI-10 (13 mg, 0.025 mmol, 1.5 eq.), COMU (10 mg, 0.024 mmol, 1.45 eq.), and N-methylmorpholine (6 µL, 0.057 mmol, 3 eq.) were dissolved in 150 µL of DMF and incubated for 1 min. SLF (9 mg, 0.017 mmol, 1 eq.) was added in 200 µL of DMF. The reaction was stirred at room temperature for 2h then the reaction was diluted to 1 mL with water/acetonitrile/formic acid (50/50/0.1). The mixture was purified by preparative HPLC and lyophilized to provide the title compound as a white powder (9.5 mg, 55%). ^{1}H NMR (500 MHz, DMSO-*d*6) δ 9.98 (s, 1H), 8.04 (t, *J* = 6.5 Hz, 1H), 7.71 (br.s, 1H), 7.46 (d, *J* = 8.0 Hz, 1H), 7.29 (t, *J* = 7.5 Hz, 1H), 7.02 (d, *J* = 7.5 Hz, 1H), 6.84 (d, *J* = 8.0 Hz, 1H), 6.81 (o, 1H), 6.76 (m, 3H), 6.68 (dd, *J* = 8.5, 1.5 Hz, 1H), 5.63 (m, 1H), 5.14 (d, *J* = 5.5 Hz, 1H), 4.45 (s, 2H), 3.72 (s, 3H), 3.71 (s, 3H), 3.71 (o, 2H), 3.67 (m, *J* = 6.3 Hz, 4H), 3.49 (br.s, 4H), 3.47 (br.s, 8H), 3.43 (t, *J* = 6.5 Hz, 2H), 3.29 (m, 2H), 3.16 (td, *J* = 13.0, 2.0 Hz, 1H), 2.67 (s, 2H), 2.53 (m, 4H), 2.23 (d, *J* = 12.0 Hz, 1H), 2.16-2.10 (m, 2H), 2.01 (m, 1H), 1.91-1.82 (m, 2H), 1.70-1.60 (m, 5H), 1.34 (m, 1H), 1.16 (s, 3H), 1.14 (s, 3H), 1.03 (d, *J* = 5.0 Hz, 1H), 0. 80 (t, *J* = 7.5 Hz, 3H). HRMS (ESI) $[M+H]^+$ for $C_{54}H_{74}ClN_4O_{14}$ 1037.4884, found 1037.4888.

Synthesis of KB02-PEG0-SLF



Scheme 4. Synthesis of KB02-PEG0-SLF

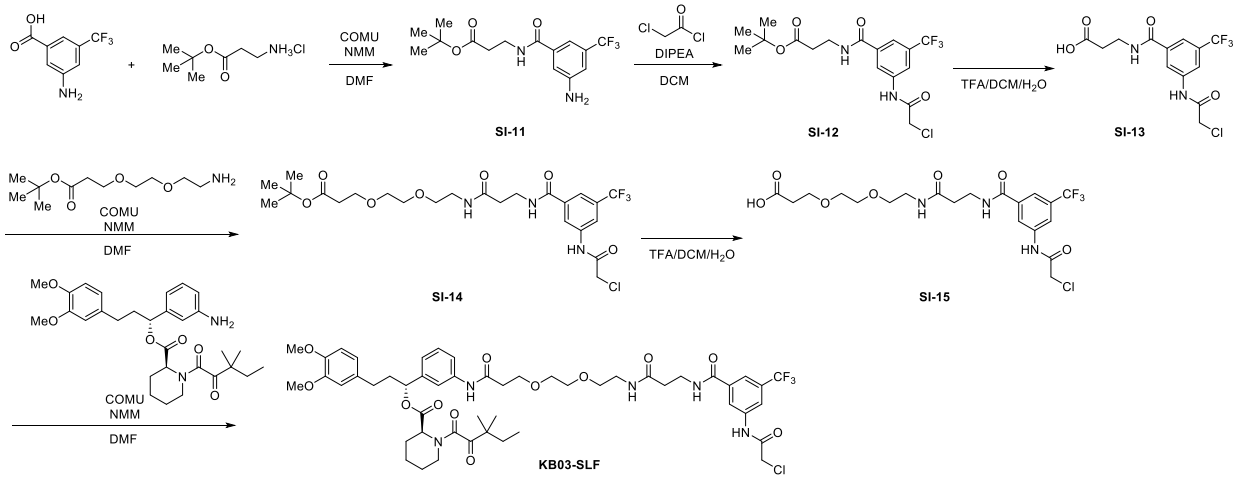
(R)-1-(3-(2-((1-(2-chloroacetyl)-1,2,3,4-tetrahydroquinolin-6-yl)oxy)acetamido)phenyl)-3-(3,4-dimethoxyphenyl)propyl (S)-1-(3,3-dimethyl-2-oxopentanoyl)piperidine-2-carboxylate (KB02-PEG0-SLF)



SI-6 (8 mg, 0.029 mmol, 1.5 eq.), COMU (12 mg, 0.028 mmol, 1.45 eq.), and N-methylmorpholine (6 µL, 0.057 mmol, 3 eq.) were dissolved in 150 µL of DMF and incubated for 1 min. SLF (10 mg, 0.019 mmol, 1 eq.) and catalytic 4-dimethylaminopyridine were added to the reaction in 200 µL of DMF. The reaction was stirred at room temperature for 2h and then diluted to 1 mL with

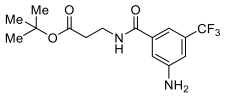
water/acetonitrile/formic acid (50/50/0.1) and purified by preparative HPLC and lyophilized to provide the title compound as a white powder (9.1 mg, 60%). ^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.39 (s, 1H), 7.67 (d, *J* = 8.5 Hz, 1H), 7.55 (s, 1H), 7.35 (t, *J* = 7.9 Hz, 1H), 7.14 – 7.10 (m, 1H), 6.91 – 6.82 (m, 2H), 6.78 (d, *J* = 8.7 Hz, 1H), 6.71 – 6.65 (m, 2H), 5.79 (dd, *J* = 8.0, 5.5 Hz, 1H), 5.33 (d, *J* = 5.7 Hz, 1H), 4.61 – 4.59 (m, 2H), 4.20 (s, 2H), 3.86 (s, 3H), 3.85 (s, 3H), 3.81 (s, 1H), 3.35 (d, *J* = 13.3 Hz, 1H), 3.15 (td, *J* = 13.1, 3.1 Hz, 1H), 2.74 (s, 2H), 2.66 – 2.52 (m, 2H), 2.36 (d, *J* = 13.8 Hz, 1H), 2.26 (dtd, *J* = 14.3, 8.9, 5.6 Hz, 1H), 2.12 – 2.04 (m, 1H), 1.78 – 1.72 (m, 1H), 1.72 – 1.67 (m, 1H), 1.67 – 1.60 (m, 1H), 1.56 (s, 5H), 1.41 – 1.33 (m, 1H), 1.21 (d, *J* = 4.2 Hz, 6H), 1.12 (d, *J* = 3.5 Hz, 1H), 0.87 (t, *J* = 7.5 Hz, 3H), 0.80 (t, *J* = 7.5 Hz, 1H). HRMS (ESI) $[M+H]^+$ for $C_{43}H_{53}ClN_3O_9$ 790.3465, found 790.3461.

Synthesis of KB03-SLF



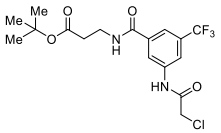
Scheme 5. Synthesis of KB03-SLF

tert-butyl 3-(3-amino-5-(trifluoromethyl)benzamido)propanoate (SI-11)



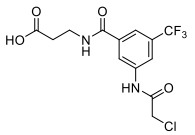
3-amino-5-(trifluoromethyl)benzoic acid (205 mg, 1 mmol, 1 eq.), COMU (428 mg, 1 mmol, 1 eq.), and N-methylmorpholine (443 µL, 4 mmol, 4 eq.) were dissolved in 400 µL of DMF and incubated for 1 min. Beta-Alanine tert-butyl ester hydrochloride (218 mg, 1.2 mmol, 1.2 eq.) was added in 600 µL of DMF and the reaction was stirred for 2h at room temperature. The reaction was diluted with ethyl acetate (10 mL), washed with water (3x10 mL), dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The residue was purified by prep TLC (dichloromethane/methanol = 9/1) to afford the title compound as a yellow solid (239 mg, 72%). ^{1}H NMR (600 MHz, Methanol-d_4) δ 7.25 (d, *J* = 2.1 Hz, 2H), 7.05 (t, *J* = 2.0 Hz, 1H), 3.58 (t, *J* = 6.9 Hz, 2H), 2.56 (t, *J* = 6.9 Hz, 2H), 1.45 (s, 9H). MS (ESI) $[M+H]^+$ for $C_{15}H_{20}F_3N_2O_3$ 333.1, found 333.1.

tert-butyl 3-(3-(2-chloroacetamido)-5-(trifluoromethyl)benzamido)propanoate (SI-12)



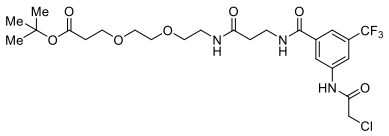
SI-11 (239 mg, 0.72 mmol, 1 eq.) and N,N-diisopropylethylamine (250 µL, 1.44 mmol, 2 eq) were dissolved in 4 mL dichloromethane and cooled to 0˚ C. Chloroacetyl chloride (86 µL, 1.1 mmol, 1.5 eq.) was added dropwise. After complete addition, the reaction was warmed to room temperature and stirred for 2h. The reaction was diluted with dichloromethane (10 mL), washed with saturated $NaHCO_3$ (15 mL), 1N HCl (15 mL), and brine (15 mL). The organic layer was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The product was purified by prep TLC (ethyl acetate/hexane = 9/1) to provide the title compound as a brown amorphous solid (230 mg, 78%). ^{1}H NMR (600 MHz, Chloroform-d) δ 8.67 (s, 1H), 8.16 (s, 1H), 8.07 (t, J = 1.8 Hz, 1H), 7.73 (s, 1H), 7.03 (t, J = 6.1 Hz, 1H), 4.21 (s, 2H), 3.69 (q, J = 6.0 Hz, 2H), 2.57 (t, J = 6.0 Hz, 2H), 1.46 (s, 9H). HRMS (ESI) $[M+H]^+$ for $C_{17}H_{20}ClF_3N_2O_4Na$ 431.0956, found 431.0960.

3-(3-(2-chloroacetamido)-5-(trifluoromethyl)benzamido)propanoic acid (SI-13)



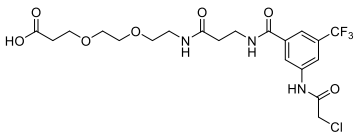
SI-12 (113 mg) was dissolved in 1 mL of dichloromethane, 1 mL of trifluoroacetic acid and 20 µL of H_2O. The reaction was stirred at room temperature for 2h then concentrated and dried under vacuum. The resulting residue was used without further purification.

tert-butyl 1-(3-(2-chloroacetamido)-5-(trifluoromethyl)phenyl)-1,5-dioxo-9,12-dioxa-2,6-diazapentadecan-15-oate (SI-14)



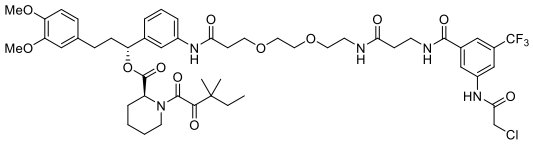
SI-13 (65 mg, 0.18 mmol, 1.2 eq.), COMU (79 mg, 0.18 mmol, 1.2 eq.), and N-methylmorpholine (51 µL, 0.46 mmol, 3 eq.) were dissolved in 333 µL of DMF and incubated for 1 min. Amino-PEG2-t-butyl ester (36 mg, 0.35 mmol, 1 eq.) was added in 667 µL of DMF and the reaction was stirred at room temperature for 1h. The reaction was diluted with ethyl acetate (10 mL) and washed with water (3x20 mL) and brine (20 mL). The organic layer was collected, dried over anhydrous $MgSO_4$, filtered and concentrated under reduced pressure. The residue was purified by flash chromatography to provide the title compound as a colorless oil (40 mg, 13%). ^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.91 – 8.85 (m, 1H), 8.24 (d, *J* = 2.1 Hz, 1H), 8.03 (t, *J* = 1.9 Hz, 1H), 7.83 – 7.80 (m, 1H), 7.63 (t, *J* = 5.7 Hz, 1H), 4.21 (s, 2H), 3.72 (t, *J* = 6.3 Hz, 2H), 3.69 (t, *J* = 6.2 Hz, 2H), 3.60 (q, *J* = 0.8 Hz, 2H), 3.57 (d, *J* = 1.8 Hz, 4H), 3.54 (ddd, *J* = 5.3, 4.4, 2.6 Hz, 2H), 2.58 – 2.52 (m, 2H), 2.48 (dt, *J* = 11.8, 6.3 Hz, 2H), 1.43 (d, *J* = 7.1 Hz, 9H). HRMS (ESI) $[M+H]^+$ for $C_{24}H_{34}ClF_3N_3O_7$ 568.2032, found 568.2036.

1-(3-(2-chloroacetamido)-5-(trifluoromethyl)phenyl)-1,5-dioxo-9,12-dioxa-2,6-diazapentadecan-15-oic acid (SI-15)



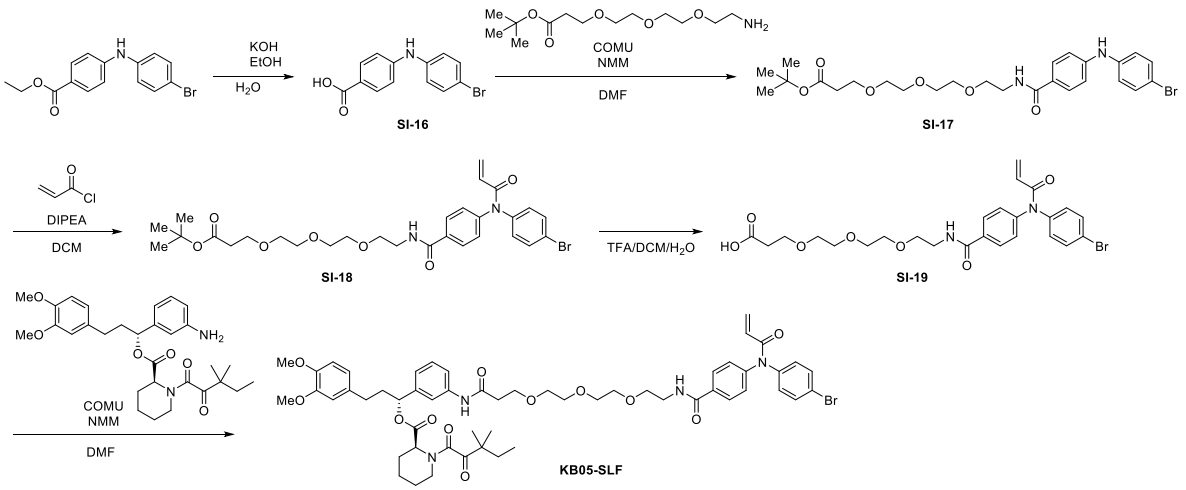
SI-14 (135 mg) was dissolved in 0.5 mL of dichloromethane, 0.5 mL of trifluoroacetic acid and 10 µL of H_2O and stirred at room temperature for 2h. The solvent was then removed under reduced pressure and dried under vacuum. The residue was used without further purification.

(R)-1-(3-(1-(3-(2-chloroacetamido)-5-(trifluoromethyl)phenyl)-1,5-dioxo-9,12-dioxa-2,6-diazapentadecan-15-amido)phenyl)-3-(3,4-dimethoxyphenyl)propyl (S)-1-(3,3-dimethyl-2-oxopentanoyl)piperidine-2-carboxylate (KB03-SLF)



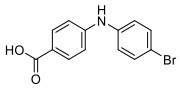
SI-15 (13 mg, 0.025 mmol, 1.5 eq.), COMU (10 mg, 0.024 mmol, 1.45 eq.) and N-methylmorpholine (6 µL, 0.057 mmol, 3 eq.) were dissolved in 150 µL of DMF and incubated for 1 min. SLF (9 mg, 0.017 mmol, 1 eq.) and catalytic 4-dimethylaminopyridine was added in 200 µL of DMF and the reaction was stirred for 2h at room temperature. The reaction was diluted up to 1 mL with water/acetonitrile/formic acid (50/50/0.1) and purified via preparative HPLC to provide the title compound as a white powder after lyophilization (9.4 mg, 55%). ^{1}H NMR (500 MHz, DMSO-*d*6) δ 10.80 (s, 1H), 9.98 (s, 1H), 8.80 (t, *J* = 5.5 Hz, 1H), 8.24 (s, 1H), 8.21 (s, 1H), 7.96 (t, *J* = 5.5 Hz, 1H), 7.91 (s, 1H), 7.70 (s, 1H), 7.46 (d, *J* = 8.0 Hz, 1H), 7.29 (t, *J* = 8.0 Hz, 1H), 7.02 (d, *J* = 7.5 Hz, 1H), 6.84 (d, *J* = 8.0 Hz, 1H), 6.76 (d, *J* = 2.0 Hz, 1H), 6.68 (dd, *J* = 8.0, 2.0 Hz, 1H), 5.63 (dd, *J* = 9.0, 5.0 Hz, 1H), 5.13 (d, *J* = 5.5 Hz, 1H), 4.30 (s, 2H), 3.72 (s, 3H), 3.71 (s, 3H), 3.66 (t, *J* = 6.5 Hz, 2H), 3.47 (br.s, 6H), 3.38 (t, *J* = 6.0 Hz, 2H), 3.18 (m, 2H), 2.54 (m, 4H), 2.38 (t, *J* = 7.0 Hz, 2H), 2.22 (d, *J* = 12.5 Hz, 1H), 2.17-2.11 (m, 2H), 2.01 (m, 1H), 1.69-1.56 (m, 5H), 1.33 (m, 1H), 1.23 (m, 1H), 1.16 (s, 3H), 1.14 (s, 3H), 1.03 (d, *J* = 5.0 Hz, 1H), 0. 80 (t, *J* = 7.0 Hz, 3H). HRMS (ESI) $[M+H]^+$ for $C_{50}H_{64}ClF_3N_5O_{12}$ 1018.4186, found 1018.4180.

Synthesis of KB05-SLF



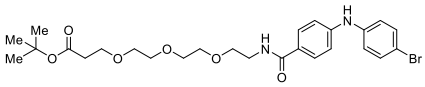
Scheme 6. Synthesis of KB05-SLF

4-((4-bromophenyl)amino)benzoic acid (SI-16)



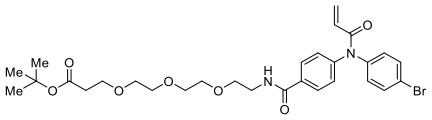
Ethyl 4-((4-bromophenyl)amino)benzoate (0.5 g, 1.5 mmol, 1 eq.) was dissolved in 7.8 mL ethanol (0.2 M). KOH (175 mg, 3 mmol, 2 eq.; in 15.6 mL water) was added and the reaction was stirred at 100 °C for 4h. The ethanol was removed and the resulting aqueous solution was cooled to 0 °C and acidified with 2N HCl to pH 2. The precipitate was collected by vacuum filtration and washed with water. The precipitate was then dissolved in ethanol and evaporated under reduced pressure to remove the remaining water. The resulting powder was used in the next step without further purification.

tert-butyl 1-(4-((4-bromophenyl)amino)phenyl)-1-oxo-5,8,11-trioxa-2-azatetradecan-14-oate (SI-17)



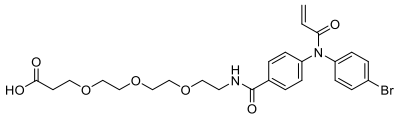
SI-16 (202 mg, 0.69 mmol, 1.2 eq.), COMU (296 mg, 0.69 mmol, 1.2 eq.), and N-methylmorpholine (190 µL, 1.7 mmol, 3 eq.) were dissolved in 1.5 mL DMF and incubated for 1 min. Amino-PEG3-t-butyl ester (160 mg, 0.58 mmol, 1 eq.) was added in 2.5 mL DMF and the reaction was stirred for 2h. The reaction was diluted with ethyl acetate (15 mL) and acidified with 1N HCl to pH 3. The organic layer was washed with water (3x 25 mL) and brine (25 mL), dried over anhydrous $MgSO_4$, and concentrated under reduced pressure. The resulting residue was purified by flash chromatography to provide the title compound as a colorless oil (215 mg, 57%). ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.66 (d, *J* = 8.7 Hz, 2H), 7.33 (d, *J* = 8.7 Hz, 2H), 6.97 (dd, *J* = 8.8, 7.2 Hz, 4H), 6.82 (d, *J* = 5.4 Hz, 1H), 3.63 (t, *J* = 6.5 Hz, 2H), 3.62 – 3.57 (m, 10H), 3.54 (td, *J* = 4.8, 4.4, 1.1 Hz, 2H), 2.43 (t, *J* = 6.5 Hz, 2H), 1.39 (s, 9H). HRMS (ESI) $[M+H]^+$ for $C_{26}H_{36}BrN_2O_6$ 551.1751, found 551.1751.

tert-butyl 1-(4-(N-(4-bromophenyl)acrylamido)phenyl)-1-oxo-5,8,11-trioxa-2-azatetradecan-14-oate (SI-18)



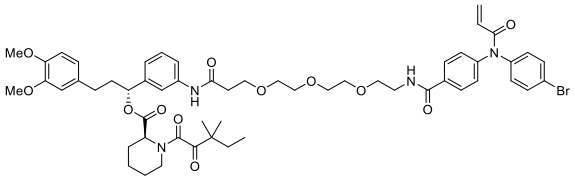
SI-17 (200 mg, 0.36 mmol, 1 eq.) and N,N-diisopropylethylamine (127 µL, 0.73 mmol, 2 eq.) were dissolved in 4 mL DCM. Acryloyl chloride (88 µL, 1.1 mmol, 3 eq.) was added dropwise at 0 °C followed by the addition of 4-dimethylaminopyridine (13 mg, 0.11 mmol, 0.1 eq.). The reaction was warmed to room temperature and stirred for 6h. The reaction was diluted with DCM (20 mL), washed with saturated $NaHCO_3$ (25 mL), 1M HCl (25 mL), water (25 mL), dried over anhydrous $MgSO_4$, and concentrated under reduced pressure. The residue was used without further purification.

1-(4-(N-(4-bromophenyl)acrylamido)phenyl)-1-oxo-5,8,11-trioxa-2-azatetradecan-14-oic acid (SI-19)



SI-18 (221 mg) was dissolved in 1 mL of dichloromethane, 1 mL of trifluoroacetic acid, and 20 µL of H_2O and stirred at room temperature for 2h. The solvent was removed under reduced pressure and dried under vacuum. The residue was used without further purification.

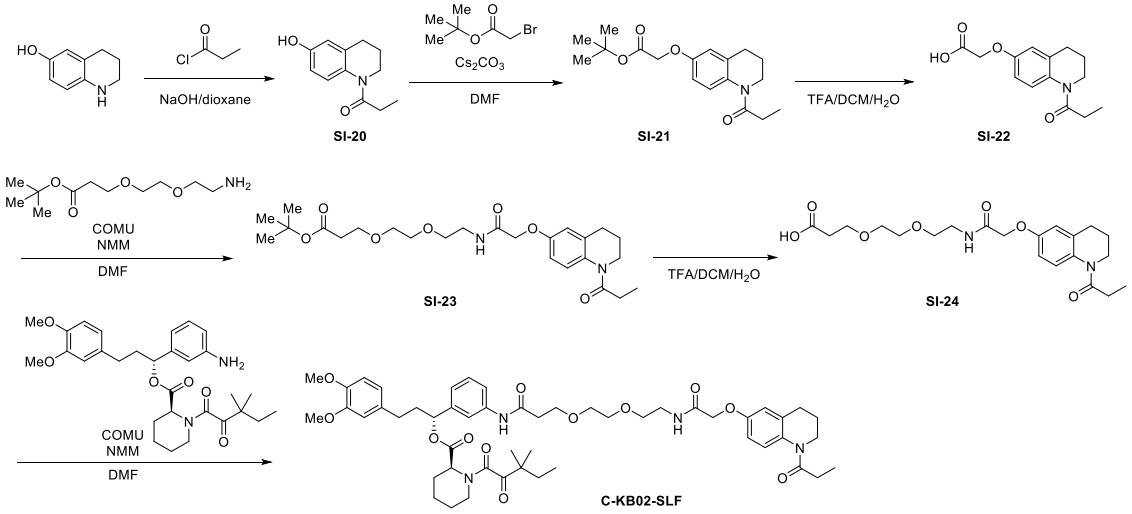
(R)-1-(3-(1-(4-(N-(4-bromophenyl)acrylamido)phenyl)-1-oxo-5,8,11-trioxa-2-azatetradecan-14-amido)phenyl)-3-(3,4-dimethoxyphenyl)propyl (S)-1-(3,3-dimethyl-2-oxopentanoyl)piperidine-2-carboxylate (KB05-SLF)



SI-19 (10 mg, 0.019 mmol, 1.5 eq.), COMU (8 mg, 0.018 mmol, 1.45 eq.), and N-methylmorpholine (4 µL, 0.037 mmol, 3 eq.) were dissolved in 150 µL of DMF in stirred for 1 min. SLF (7 mg, 0.012 mmol, 1 eq.) and catalytic 4-dimethylaminopyridine were added in 200 µL of DMF and the reaction was stirred at room temperature for 4h. The reaction solution was diluted to 1 mL with water/acetonitrile/formic acid (50/50/0.1) and purified by preparative HPLC to provide the title compound as a white powder after lyphilization (3.7 mg, 28%). ^{1}H NMR (500 MHz, DMSO-*d*6) δ 9.99 (s, 1H), 8.55 (t, *J* = 5.0 Hz, 1H), 7.87 (d, *J* = 8.0 Hz, 2H), 7.70 (s, 1H), 7.61 (d, *J* = 8.0 Hz, 2H), 7.45 (d, *J* = 8.0 Hz, 1H), 7.35 (d, *J* = 8.0 Hz, 2H), 7.30-7.20 (m, 3H), 7.01 (d, *J* = 7.5 Hz, 1H), 6.84 (d, *J* = 8.0 Hz, 1H), 6.75 (s, 1H), 6.67 (d, *J* = 8.0 Hz, 1H), 6.25 (d, *J* = 17.0, 1H), 6.12

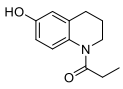
(dd, *J* = 17.0, 10.0 Hz, 1H), 5.71 (d, *J* = 10.0, 1H), 5.63 (m, 1H), 5.12 (d, *J* = 4.5, 1H), 3.71 (s, 3H), 3.70 (s, 3H), 3.66 (t, *J* = 6.0 Hz, 2H), 3.48 (m, 12H), 3.15 (m, 2H), 2.53 (m, 3H), 2.21 (d, *J* = 13.5 Hz,1H), 2.12 (m, 1H), 2.00 (m, 1H), 1.69-1.55 (m, 5H), 1.34 (m, 1H), 1.21 (m, 1H), 1.16 (s, 3H), 1.13 (s, 3H), 1.03 (d, *J* = 5.0 Hz, 1H), 0.79 (t, *J* = 7.5 Hz, 3H). HRMS (ESI) $[M+H]^+$ for $C_{55}H_{68}BrN_4O_{12}$ 1055.4011, found 1055.4009.

Synthesis of inactive control C-KB02-SLF



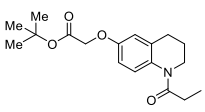
Scheme 7. Synthesis of C-KB02-SLF

1-(6-hydroxy-3,4-dihydroquinolin-1(2H)-yl)propan-1-one (SI-20)



1,2,3,4-tetrahydroquinolin-6-ol (1 g, 6.7 mmol, 1 eq.) and NaOH (0.32 g, 8.0 mmol, 1.2 eq.) were dissolved in water/dioxane (1:1, 20 mL) at 0 ºC. Propanoyl chloride (0.64 mL, 7.4 mmol, 1.1 eq.) was added dropwise and the reaction was warmed to room temperature and stirred for 4h. The reaction was acidified with 1N HCl (pH < 4) then extracted with ethyl acetate (2x20mL). The combined organic layers were washed with brine (100 mL), dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The resulting residue was used for the next step without purification.

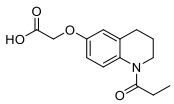
tert-butyl 2-((1-propionyl-1,2,3,4-tetrahydroquinolin-6-yl)oxy)acetate (SI-21)



SI-20 (0.95 g, 4.6 mmol, 1 eq.) and Cs_2CO_3 (2.3 g, 6.9 mmol, 1.5 eq.) were dissolved in 15 mL DMF. Tert-Butyl bromoacetate (0.78 mL, 5.8 mmol, 1.25 eq.) was added dropwise and the reaction was stirred at room temperature for 3h. The reaction was diluted with ethyl acetate (30 mL) and acidified with 1N HCl to pH <4. The organic layer was washed with water (3x40mL), dried

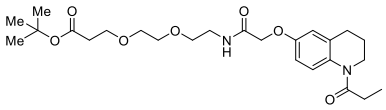
over anhydrous Na_2SO_4, and concentrated under reduced pressure. The residue was purified by flash chromatography (10-30 % ethyl acetate/hexane) to provide the title compound as a yellow amorphous solid (815 mg, 55%). ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.16 – 6.86 (m, 1H), 6.70 – 6.64 (m, 2H), 4.47 (s, 2H), 3.77 – 3.69 (m, 2H), 2.64 (t, *J* = 6.6 Hz, 2H), 2.44 (q, *J* = 7.4 Hz, 2H), 1.90 (p, *J* = 6.8 Hz, 2H), 1.47 (s, 9H), 1.11 (t, *J* = 7.4 Hz, 3H). HRMS (ESI) $[M+H]^+$ for $C_{18}H_{26}NO_4$ 320.1856, found 320.1859.

2-((1-propionyl-1,2,3,4-tetrahydroquinolin-6-yl)oxy)acetic acid (SI-22)



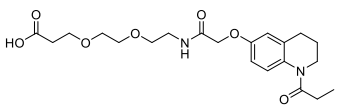
SI-21 (442 mg) was dissolved in 2 mL of dichloromethane, 2 mL of trifluoroacetic acid and, 40 µL of H_2O and stirred at room temperature for 2h. The solvent was then removed and dried under vacuum. The resulting residue was used without further purification.

tert-butyl 3-(2-(2-(2-((1-propionyl-1,2,3,4-tetrahydroquinolin-6-yl)oxy)acetamido)ethoxy)ethoxy)propanoate (SI-23)



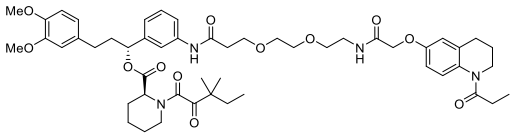
SI-22 (270 mg, 1.03 mmol, 1.2 eq.), COMU (440 mg, 1.03 mmol, 1.2 eq.), and N-methylmorpholine (283 µL, 2.57 mmol, 3 eq.) were dissolved in 1.5 mL of DMF and stirred for 1 min. Amino-PEG2-t-butyl ester (200 mg, 0.86 mmol, 1 eq.) was added in 2.5 mL of DMF and the reaction was stirred at room temperature for 1 h. The reaction was diluted with ethyl acetate (10mL) and the organic phase washed with water (3x15mL) and brine (1x15mL). The organic layer was separated, dried over anhydrous $MgSO_4$, and concentrated. The residue was purified by flash chromatography (DCM/EtOAc; 1:1) to provide the title compound as a colorless oil (212 mg, 43%). ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.01 (t, *J* = 5.7 Hz, 1H), 6.71 (dt, *J* = 8.7, 2.4 Hz, 1H), 6.68 (s, 1H), 4.43 (s, 2H), 3.74 – 3.69 (m, 2H), 3.67 (td, *J* = 6.4, 2.0 Hz, 2H), 3.55 (d, *J* = 2.3 Hz, 4H), 3.51 (ddd, *J* = 6.5, 3.4, 1.5 Hz, 2H), 2.65 (s, 2H), 2.48 – 2.40 (m, 4H), 2.18 (qd, *J* = 7.6, 2.1 Hz, 1H), 1.90 (td, *J* = 6.6, 1.8 Hz, 2H), 1.41 (d, *J* = 1.5 Hz, 2H), 1.40 (d, *J* = 2.3 Hz, 9H), 1.11 (td, *J* = 7.8, 2.6 Hz, 3H). HRMS (ESI) $[M+Na]^+$ for $C_{25}H_{38}N_2O_7Na$ 501.2571, found 501.2580.

3-(2-(2-(2-((1-propionyl-1,2,3,4-tetrahydroquinolin-6-yl)oxy)acetamido)ethoxy)ethoxy)propanoic acid (SI-24)



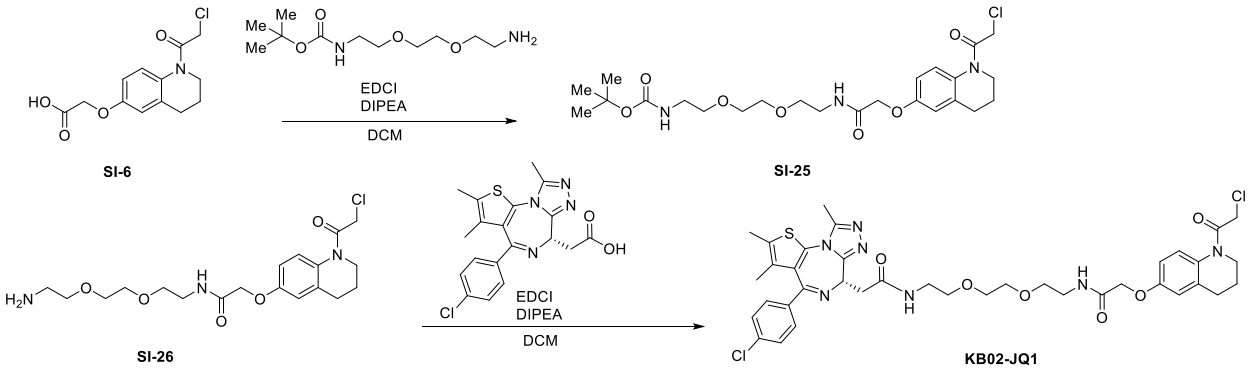
SI-23 (AMT) was dissolved in 0.5 mL of dichloromethane, 0.5 mL of trifluoroacetic acid, and 10 µL of H_2O and stirred at room temperature for 2h. The reaction was then concentrated under reduced pressure and dried under vacuum. The resulting residue was used without further purification.

(R)-3-(3,4-dimethoxyphenyl)-1-(3-(3-(2-(2-(2-((1-propionyl-1,2,3,4-tetrahydroquinolin-6-yl)oxy)acetamido)ethoxy)ethoxy)propanamido)phenyl)propyl (S)-1-(3,3-dimethyl-2-oxopentanoyl)piperidine-2-carboxylate (C-KB02-SLF)



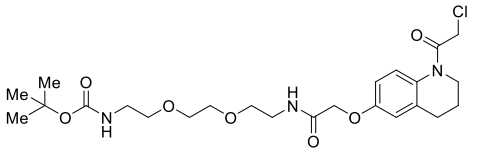
SI-24 (12 mg, 0.029 mmol, 1.5 eq.), COMU (12 mg, 0.028 mmol, 1.45 eq.), and N-methylmorpholine (6 µL, 0.057 mmol, 3 eq.) were dissolved in 150 µL of DMF and stirred for 1 min. SLF (10 mg, 0.019 mmol, 1 eq.) was added in 200 µL of DMF and the reaction was stirred for 4h at room temperature. The reaction was diluted to 1 mL with water/acetonitrile/formic acid (50/50/0.1) and purified via preparative HPLC then lyophilized to provide the title compound as a white powder (8.4 mg, 47%). ^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.53 (s, 1H), 7.54 (d, *J* = 13.2 Hz, 2H), 7.28 (d, *J* = 7.9 Hz, 1H), 7.04 (t, *J* = 6.5 Hz, 1H), 6.95 (d, *J* = 6.2 Hz, 1H), 6.77 (dd, *J* = 8.4, 4.4 Hz, 1H), 6.72 (dd, *J* = 8.7, 3.0 Hz, 1H), 6.67 (dq, *J* = 4.5, 2.1 Hz, 3H), 5.77 (dd, *J* = 8.0, 5.4 Hz, 1H), 5.31 (d, *J* = 5.7 Hz, 1H), 4.43 (s, 2H), 3.86 (s, 3H), 3.84 (s, 3H), 3.82 (t, *J* = 5.9 Hz, 2H), 3.73 (s, 2H), 3.66 (d, *J* = 4.6 Hz, 3H), 3.60 (t, *J* = 5.3 Hz, 2H), 3.51 (q, *J* = 5.5 Hz, 2H), 3.34 (d, *J* = 13.5 Hz, 1H), 3.14 (td, *J* = 13.1, 3.1 Hz, 1H), 2.67 (s, 2H), 2.63 (t, *J* = 5.7 Hz, 1H), 2.56 (ddd, *J* = 18.6, 9.9, 6.0 Hz, 1H), 2.47 (q, *J* = 7.4 Hz, 2H), 2.35 (d, *J* = 13.7 Hz, 1H), 2.22 (ddd, *J* = 13.8, 11.5, 7.1 Hz, 1H), 2.10 – 2.01 (m, 1H), 1.93 (p, *J* = 6.5 Hz, 2H), 1.73 – 1.69 (m, 1H), 1.69 – 1.65 (m, 1H), 1.65 – 1.62 (m, 1H), 1.58 (s, 6H), 1.45 (ddt, *J* = 12.6, 8.6, 3.8 Hz, 1H), 1.36 (d, *J* = 13.0 Hz, 1H), 1.22 (d, *J* = 5.8 Hz, 5H), 1.15 (t, *J* = 7.4 Hz, 3H), 1.11 (d, *J* = 2.3 Hz, 1H), 0.89 (t, *J* = 7.5 Hz, 2H), 0.80 (t, *J* = 7.5 Hz, 1H). HRMS (ESI) $[M+H]^+$ for $C_{58}H_{69}N_4O_{12}$ 929.4906, found 929.4902.

Synthesis of KB02-JQ1



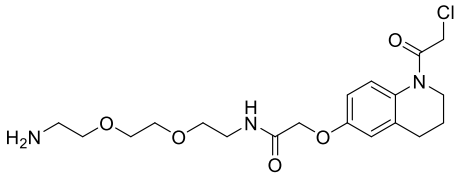
Scheme 8. Synthesis of KB02-JQ1

tert-butyl (2-(2-(2-(2-((1-(2-chloroacetyl)-1,2,3,4-tetrahydroquinolin-6-yl)oxy)acetamido)ethoxy)ethoxy)ethyl)carbamate (SI-25)



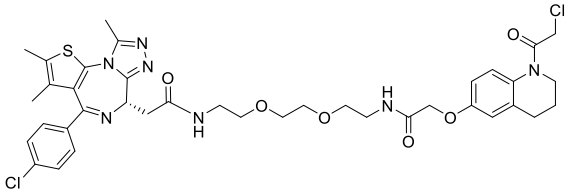
SI-6 (100mg, 0.35 mmol, 1 eq.), t-Boc-N-amido-PEG2-amine (96 mg, 0.38 mmol, 1.1 eq.), EDCI (81 mg, 0.42, 1.2 eq.), N,N-diisopropylethylamine (122 µL, 0.7 mmol, 2 eq.), and catalytic 4-dimethylaminopyridine were dissolved in DCM (3 mL) and stirred at room temperature for 4 h. The reaction was diluted with DCM (10 mL) and washed with 1N HCl (15 mL), saturated $NaHCO_3$ (15 mL), and brine (15 mL). The organic layer was dried over anhydrous Na_2SO_4 and concentrated. The residue was purified via flash chromatography (EtOAc/DCM, 1:1) to provide the title compound as a colorless oil (31 mg,17%) ^{1}H NMR (500 MHz, Chloroform-*d*) δ 6.99 (s, 1H), 6.78 (dd, *J* = 8.7, 2.7 Hz, 1H), 6.75 (s, 1H), 5.01 (d, *J* = 7.1 Hz, 1H), 4.48 (s, 2H), 4.18 (s, 2H), 3.79 (t, *J* = 6.7 Hz, 2H), 3.59 (s, 6H), 3.56 (dd, *J* = 10.7, 5.8 Hz, 2H), 3.53 (t, *J* = 5.3 Hz, 2H), 3.29 (t, *J* = 5.5 Hz, 2H), 2.70 (s, 2H), 1.98 (s, 2H), 1.42 (s, 9H). HRMS (ESI) $[M+H]^+$ for $C_{24}H_{37}ClN_3O_7$ 514.2314, found 514.2310.

N-(2-(2-(2-aminoethoxy)ethoxy)ethyl)-2-((1-(2-chloroacetyl)-1,2,3,4-tetrahydroquinolin-6-yl)oxy)acetamide (SI-26)



4N HCl in dioxane (1.25 mL, 10 eq.) was added to SI-25 (31 mg, 0.05 mmol) at 0°C. The reaction was warmed to room temperature and stirred for 4h. The solvent was removed under reduced pressure and dried under vacuum. The resulting residue was used without further purification.

(S)-2-((1-(2-chloroacetyl)-1,2,3,4-tetrahydroquinolin-6-yl)oxy)-N-(2-(2-(2-(2-(4-(4-chlorophenyl)-2,3,9-trimethyl-6H-thieno[3,2-f][1,2,4]triazolo[4,3-a][1,4]diazepin-6-yl)acetamido)ethoxy)ethoxy)ethyl)acetamide (KB02-JQ1)



(+)-JQ1 acid (4 mg, 0.01 mmol, 1 eq.), EDCI (3 mg, 0.01 mmol, 1.2 eq.), catalytic 4-dimethylaminopyridine, and N,N-diisopropyethylamine (10µL, 0.05 mmol, 5eq.) were added to a solution of SI-26 (9 mg, 0.02 mmol, 2 eq.) in DCM (0.5 mL) and stirred at room temperature for 2h. The reaction was diluted with DCM (10 mL) and washed with 1N HCl (15 mL), saturated $NaHCO_3$ (15 mL), and brine (15 mL). The organic layer was then dried over anhydrous Na_2SO_4 and concentrated. The residue was purified by prep TLC (DCM/MeOH; 19:1) to provide the title

compound as an off white powder (4 mg, 47%). ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.43 – 7.39 (m, 2H), 7.35 – 7.30 (m, 2H), 6.89 (s, 1H), 6.76 (d, *J* = 9.9 Hz, 2H), 4.63 (dd, *J* = 7.6, 6.5 Hz, 1H), 4.50 (s, 2H), 4.17 (s, 2H), 3.78 (s, 2H), 3.66 – 3.62 (m, 6H), 3.61 – 3.56 (m, 4H), 3.54 (d, *J* = 7.6 Hz, 1H), 3.49 (q, *J* = 5.4 Hz, 2H), 3.37 – 3.32 (m, 1H), 2.66 (d, *J* = 6.7 Hz, 3H), 2.40 (dd, *J* = 1.4, 0.8 Hz, 3H), 1.71 – 1.65 (m, 8H). HRMS (ESI) $[M+H]^+$ for $C_{38}H_{44}Cl_2N_7O_6S$ 796.2445, found 796.2448.

References

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