

Supplementary Material

DETERMINATION OF ANTIBODY-DRUG CONJUGATE RELEASED PAYLOAD SPECIES USING DIRECTED IN VITRO ASSAYS AND MASS SPECTROMETRIC INTERROGATION

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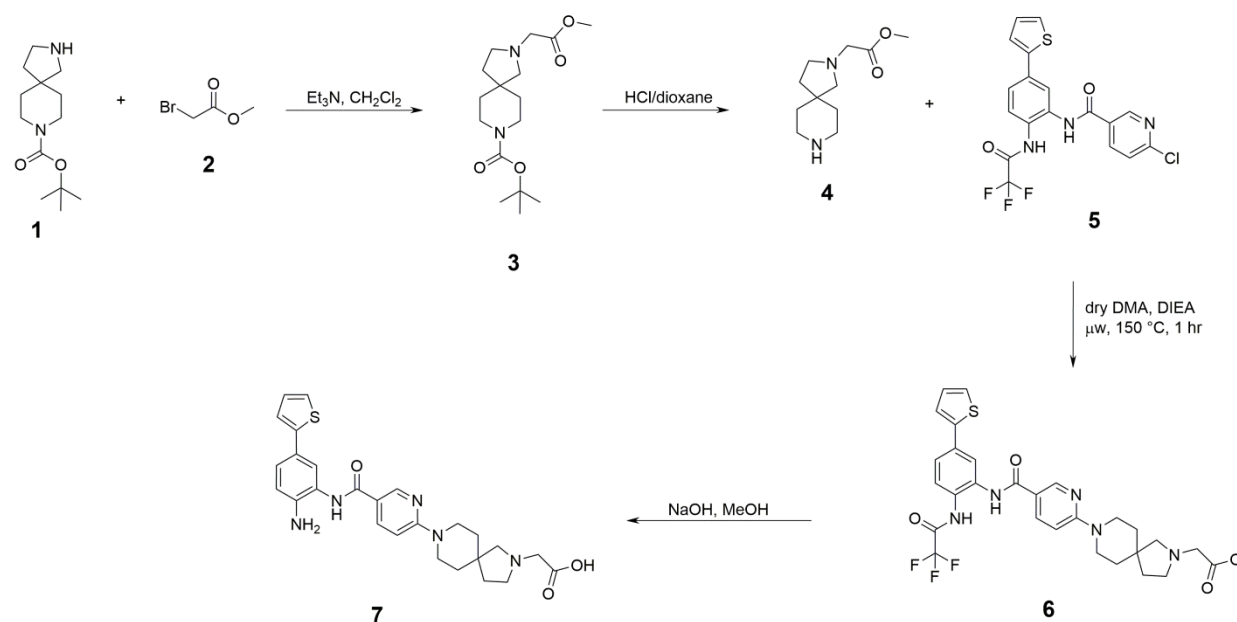
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General methods for chemical synthesis

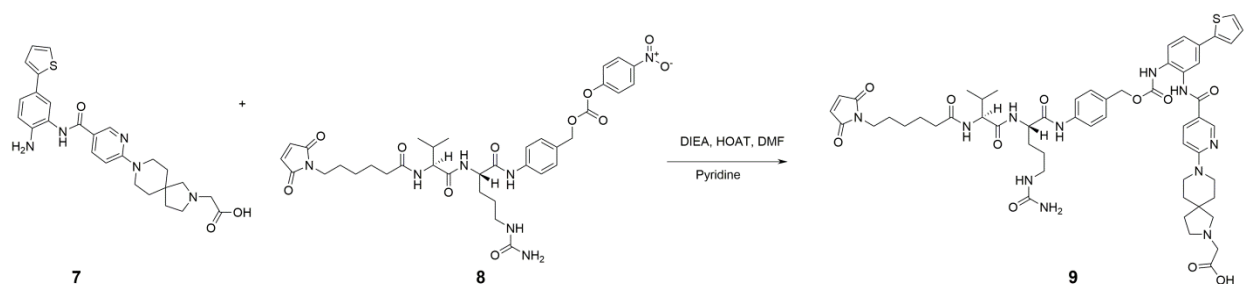
Starting materials and reagents were purchased from Aldrich or VWR and used as received. Analytical thin-layer chromatography (TLC) was performed on glass plates, precoated with silica gel. Flash chromatography was carried out using silica gel 60 (Redisep) as the stationary phase. Analytical HPLC was performed on reversed phase-HPLC (RP-HPLC) instruments, using C18 columns and a binary solvent system (MeCN and H₂O with 0.1% CF₃CO₂H). ¹H NMR spectra were recorded on Varian 400 MHz or Bruker 500 MHz spectrometers. Chemical shifts are reported in ppm relative to the signals corresponding to the residual non-deuterated solvents (CD₃OD: δ 3.31 ppm and DMSO-*d*₆: δ 2.50 ppm / 298 K).

Synthesis of mc-Val-Cit-PABC-Spirocyclic Nicotinamide



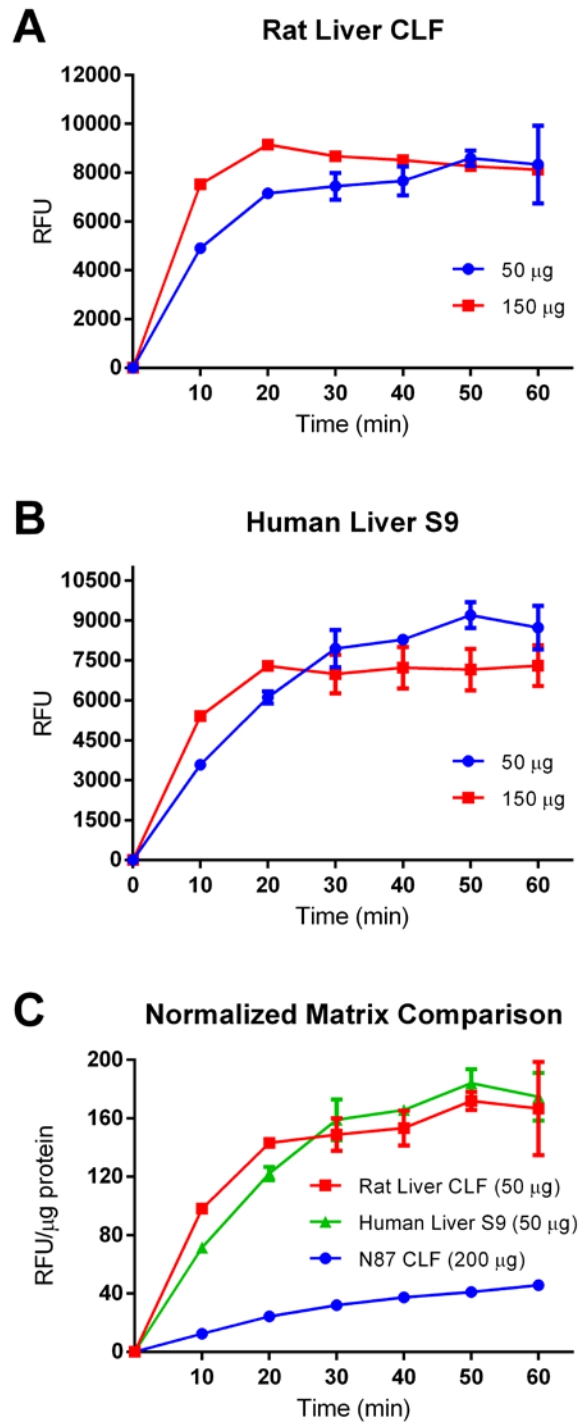
Spirocyclic Nicotinamide Intermediate 7: To a solution of compound 1 (5.0 g, 20.8 mmol) in 60 mL of dry DCM was added Et₃N (6.32 g, 62.4 mmol) and compound 2 (4.14 g, 27.0 mmol). The mixture was stirred at 10 – 20 °C for 16 h. The reaction mixture was added to water (200 mL) and extracted with DCM (3 x 300 mL). The organic layer was washed with water (2 x 150 mL), and concentrated to give crude product, which was purified by ISCO flash chromatography on silica gel (eluent: DCM:MeOH, from pure 100/0 to 10/1) to provide desired product compound 3 (5.2 g, 80 %) as a yellow oil [LRMS ESI *m/z* (M+H: 313.2)]. To a solution of compound 3 (5.2 g, 16.6 mmol) in 20 mL of dry dioxane was added 4 M HCl/dioxane (20 mL). The reaction mixture was stirred at 10 – 20 °C for 4 h. The reaction mixture was concentrated to give desired product compound 4 (4.80 g, 100%) as a yellow solid [LRMS ESI *m/z* (M+H: 213.1)]. To a solution of compound 5 (200.0 mg, 0.470 mmol) in 8 mL of DMF was added DIEA (364 mg, 2.82 mmol) and compound 4 (199 mg, 0.939 mmol) under N₂. The reaction mixture was stirred and heated at 150 °C for 3 h in microwave. The reaction mixture was concentrated and the residue was added to water (100 mL) and extracted with DCM (3 x 200 mL). The organic layer was washed with water (80 mL), and concentrated to give crude product,

which was purified by ISCO flash chromatography on silica gel (eluent: DCM:MeOH, from pure 100/0 to 10/1) to provide desired product compound **6** (280 mg, 79%) as a yellow oil [LRMS ESI m/z (M+H: 601.9)]. Compound **6** (0.040 g, 0.066 mmol) was diluted with ~5 mL of methanol in a vial, treated with ~1 mL of 1 M NaOH, capped, and stirred at 40 °C for 3 h. The reaction solution was adsorbed onto silica gel and purified by normal phase chromatography on silica gel eluting with 0 – 100% MeOH in DCM to afford compound **7** upon concentration as a tan solid (0.030 g, 94%). LRMS ESI m/z (M+H: 492.0). ^1H NMR (400 MHz, CD_3OD) δ 8.70 – 8.84 (m, 1H), 8.06 – 8.20 (m, 1H), 7.78 – 7.87 (m, 1H), 7.43 – 7.54 (m, 1H), 7.33 – 7.41 (m, 1H), 7.22 (br. s., 2H), 6.98 – 7.06 (m, 1H), 6.78 – 6.95 (m, 2H), 3.75 (br. s., 6H), 3.48 – 3.61 (m, 2H), 3.40 (m, 2H), 2.09 (m, 2H), 1.79 (br. s., 4H).

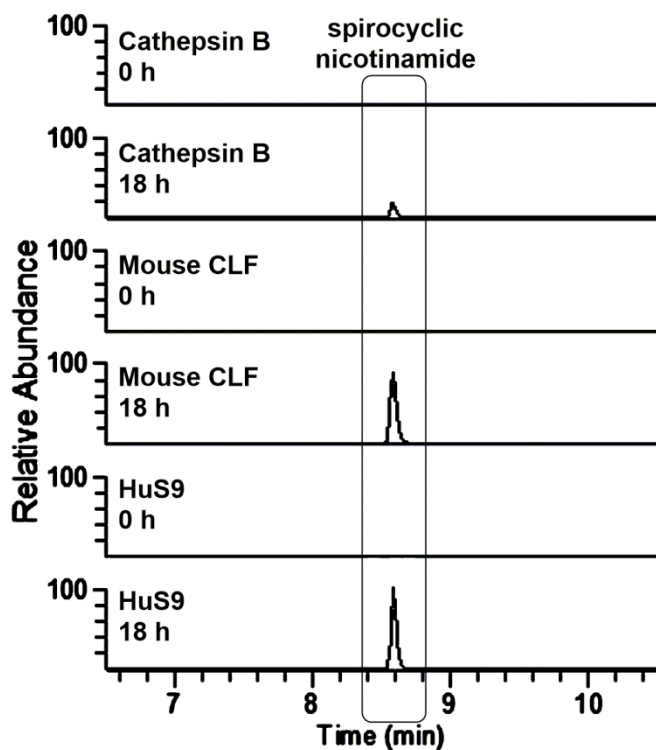


mc-Val-Cit-PABC-Spirocyclic Nicotinamide (9). The spirocyclic nicotinamide intermediate **7** (0.27 g, 0.55 mmol) was combined with HOAT (0.20 g, 1 mmol), DIEA, (0.02 g, 0.1 mmol), and pyridine (0.02 g, 0.1 mmol) in a vial, diluted with 3.0 mL of dry DMA (sure seal bottle) and treated with the nitro carbonate compound **8** (1.0 g, 1.4 mmol). The vial was capped and the resultant reaction solution was stirred at 50 °C for 2 h. The reaction solution was then cooled to ambient temperature and triturated with MTBE (3 x 15 mL). The residue recovered was purified first by normal phase chromatography on a 24 gram column eluting with 0 – 100% MeOH in DCM. The material recovered was then further purified by reverse phase chromatography on a Sunfire C18 4.6 x 50 mm 5 μm column eluting with acetonitrile/water each containing 0.05% TFA. The desired material **9** was recovered as a white solid (0.2 g, 33% yield). LRMS (ESI) m/z 1090.6 (M+H), 545.9 (1/2M+); ^1H NMR (600 MHz, 1/1 $\text{CD}_3\text{OD}/\text{DMSO}-d_6$) δ 9.74 (s, 1H), 8.66 (br. s., 1H), 8.15 (d, J = 7.63 Hz, 1H), 8.01 (dd, J = 8.80, 1.76 Hz, 1H), 7.87 (s, 1H), 7.56 (d, J = 8.22 Hz, 2H), 7.48 – 7.53 (m, 2H), 7.36 – 7.40 (m, 2H), 7.33 (d, J = 8.22 Hz, 2H), 7.06 – 7.11 (m, 1H), 6.91 (d, J = 9.39 Hz, 1H), 6.76 (s, 2H), 5.16 (s, 2H), 4.48 – 4.56 (m, 1H), 4.23 (s, 2H), 4.12 (d, J = 7.63 Hz, 1H), 3.79 – 3.85 (m, 3H), 3.66 – 3.74 (m, 3H), 3.45 (t, J = 7.04 Hz, 2H), 3.17 – 3.26 (m, 2H), 3.06 – 3.14 (m, 2H), 2.28 (t, J = 7.34 Hz, 2H), 2.11 – 2.17 (br.s., 2H), 2.04 – 2.11 (m, 2H), 1.87 – 1.95 (m, 2H), 1.71 – 1.85 (m, 6H), 1.51 – 1.67 (m, 7H), 1.26 – 1.34 (m, 3H), 0.98 (d, J = 5.28 Hz, 6H).

Supplemental Figure S1. Relative activity of liver preparations as assessed by cleavage of the probe substrate Z-RR-AMC at pH 5.0. (A) Rat liver CLF at two protein concentrations; (B) Human liver S9 fraction at two concentrations; (C) Rat liver CLF, human liver S9 fraction, and N87 CLF, normalized for protein content.



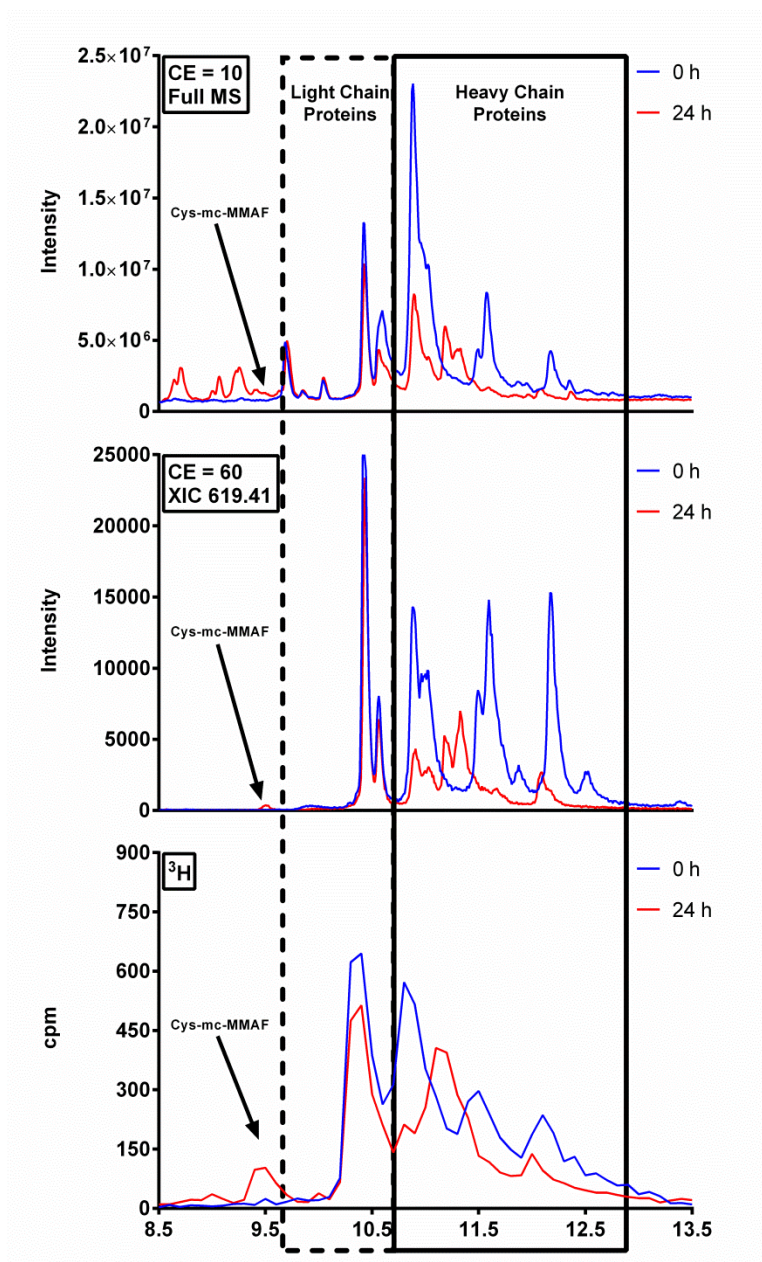
Supplemental Figure S2. The release of the spirocyclic nicotinamide payload from cysteine-conjugated **ADC III** containing the mc-vc-PABC cleavable linker. By 18 h, the release of the spirocyclic nicotinamide product (m/z 492.206) was observed in incubations with purified cathepsin B, mouse liver CLF, and human liver S9 fraction. The expected product was monitored by targeted MS^2 of m/z 492.21, and no other products were observed by examination of the full scan MS data.



Mass spectrum of compound 10 showing relative abundance versus m/z . The base peak is at m/z 619.4064. Other significant peaks are labeled at m/z 335.1973, 400.1901, 428.1850, 457.2696, 516.3069, 587.3805, 680.3648, 822.0934, 892.5521, 996.5421, and 1014.5577.



Supplemental Figure S4. Mass chromatograms of noncleavable cysteine **ADC II** after 0 hr (blue trace) and 24 h (red trace) of incubation time with mouse liver CLF. The heavy chain peaks are highly digested by 24 h, but the light chain peaks are largely unchanged (top). The XIC of m/z 619.41 (middle pane) shows peaks which contain the MMAF payload, and compares well with radiochromatograms (bottom pane) from incubations with [^3H]**ADC II**.



Supplemental Figure S5. CID spectrum of the spirocyclic nicotinamide payload product of lysosomal release from **ADC III**. The intense fragment ion at m/z 302.15 can be observed.

