

Selective USP7 inhibition elicits cancer cell killing through a p53-dependent mechanism

Nathan J. Schauer^{1,2,†}, Xiaoxi Liu^{1,2,†}, Robert S. Magin^{1,2}, Laura M. Doherty^{1,2,3}, Wai Cheung Chan^{1,2}, Scott B. Ficarro^{1,13}, Wanyi Hu¹, Rebekka M. Roberts¹, Roxana E. Iacob⁴, Björn Stolte^{5,6,7}, Andrew O. Giacomelli^{7,8,9}, Sumner Perera¹⁰, Kyle McKay¹¹, Sarah A. Boswell³, Ellen L. Weisberg⁸, Arghya Ray^{8,12}, Dharminder Chauhan^{8,12}, Sirano dhe Paganon¹, Ken C. Anderson^{8,12}, James D. Griffin⁸, Jianing Li¹¹, William C. Hahn^{7,8,9}, Peter K. Sorger³, John R. Engen⁴, Kimberly Stegmaier^{5,7}, Jarrod A. Marto^{1,13}, Sara J. Buhrlage^{1,2,*}

¹ Department of Cancer Biology and the Linde Program in Cancer Chemical Biology, Dana-Farber Cancer Institute, Boston, MA, USA

² Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA, USA

³ Department of Systems Biology and Laboratory of Systems Pharmacology, Harvard Medical School, Boston, MA, USA

⁴ Department of Chemistry and Chemical Biology, Northeastern University, Boston, MA, USA

⁵ Department of Pediatric Oncology, Dana-Farber Cancer Institute and Boston Children's Hospital, Boston, MA, USA

⁶ Dr. von Hauner Children's Hospital, Department of Pediatrics, University Hospital, LMU Munich, Munich, Germany

⁷ The Broad Institute of MIT and Harvard University, Cambridge, MA, USA

⁸ Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, MA, USA

⁹ Department of Medicine, Harvard Medical School, Boston, MA, USA

¹⁰ Harvard College, Cambridge, MA, USA

¹¹ Department of Chemistry, University of Vermont, Burlington, VT, USA

¹² The LeBow Institute for Myeloma Therapeutics and Jerome Lipper Myeloma Center, Dana-Farber Cancer Institute, Boston, MA, USA

¹³ Department of Oncologic Pathology and Blais Proteomics Center, Dana-Farber Cancer Institute, Boston, MA, USA

† These authors contributed equally to this work

* e-mail: saraj_buhrlage@dfci.harvard.edu

Supplementary Data

Supplementary Figures S1 – S16

Supplementary Synthetic Methods

Supplementary Figure Captions

Figure S1: Full-length recombinant purified USP7 was pre-incubated with XL041 at the indicated concentrations for the indicated times, then Ub-AMC was added and fluorescence was monitored. Graphpad Prism software was used to calculate initial reaction velocity and derive K_I / k_{inact} values (bottom panel).

Figure S2: Mass spectra (left) and zero-charge mass spectra (right) of WT USP7 catalytic domain incubated for 4 hours with DMSO (top) or 2.5-fold molar excess XL058 (bottom).

Figure S3: Mass spectra (left) and zero-charge mass spectra (right) of USP7 C223A catalytic domain incubated for 2 hours with DMSO (top) or 2.5-fold molar excess XL177A (bottom).

Figure S4: Representative flow cytometry gating for cell cycle analysis of MCF7 cells treated with DMSO, serum starvation, 1 μ M taxol, 1 μ M XL177A, or 1 μ M XL177B for 24 hours. Each plot is one of three experimental replicates for each condition.

Figure S5: MCF7 cells were transfected with the indicated pcDNA constructs for 48 hours, then treated with XL177A at the indicated concentration for 3 hours. Cells were lysed, and FLAG-USP7 target engagement was assessed with the DUB ABP HA-Ub-VS. Densitometry was performed using ImageStudio software and analyzed using Graphpad Prism.

Figure S6: HDX data. **A.** USP7 coverage map showing the common peptic peptides that were compared between free USP7 and XL041 USP7 bound, and the deuterium incorporation plots for all 81 peptic peptides that were compared between USP7 free (red) and XL041 bound (blue). On the x axis is the time in minutes and on the y-axis is the relative uptake (Da). The maximum y-axis value for each plot represents the theoretical maximum amount of D that can be incorporated into a peptide. Values represent the mean of two individual measurements; error bars, s.d. **B.** USP7 coverage map showing all the common peptic peptides that were compared between free USP7 and XL177A USP7 bound, and the deuterium incorporation plots for all 105 peptic peptides that were compared between USP7 free (red) and XL177A bound (blue). **C.** Chiclet representation of the HDX MS data. The peptides from panel A and B are represented from N to C terminus (top to bottom) at each time point (left to right). The deuterium level for each bound state was subtracted from

that of the protein alone and colored according to the legend shown. The horizontal dotted lines are placed to help orient the readers to notice the regions of difference between the two states. Note that in the HDX MS data set the protein sequence numbering is different and Cys34 is actually Cys223.

Figure S7. Snapshot of the XL188-USP7 construct (red) and the XL177A-USP7 construct (orange) superimposed on each other after 150 ns of simulation time shows the benzyl moiety of XL177A in a similar confirmation to that of XL188.

Figure S8: DUBs identified by ABPP on HEK293 lysate when probed with 1 μ M of the indicated probe, with a 1% FDR cut-off.

Figure S9: Heatmaps showing correlation between XL177A and XL177B overall viability among all cell lines screened in PRISM at the indicated doses. XL177A = K87132179; XL177B = K79243224

Figure S10: PRISM results from XL177B (top) and Nutlin-3A (bottom) showing correlations with CRISPR profiling (left), mutational profiling (center), and lineage enrichment (right).

Figure S11: Cellular proliferation of panels of acute myeloid leukemia (AML); soft tissue sarcomas including two rhabdoid (G401, G402) and one uterine sarcoma (MES-SA); and multiple myeloma cell lines treated with XL177A (left) or XL177B (right). Red data points indicated *TP53*-WT cell lines, and black data points indicate *TP53* mutants.

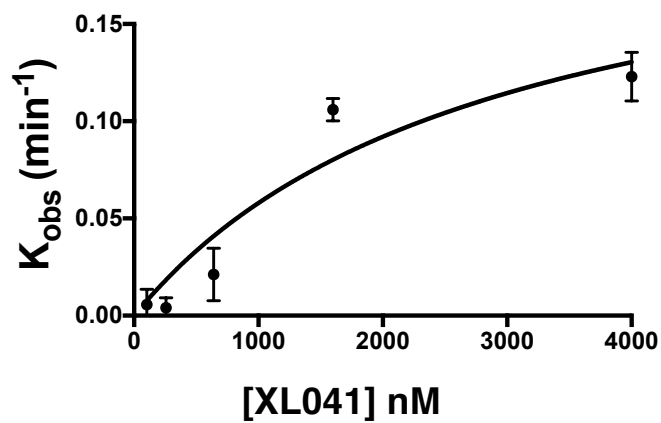
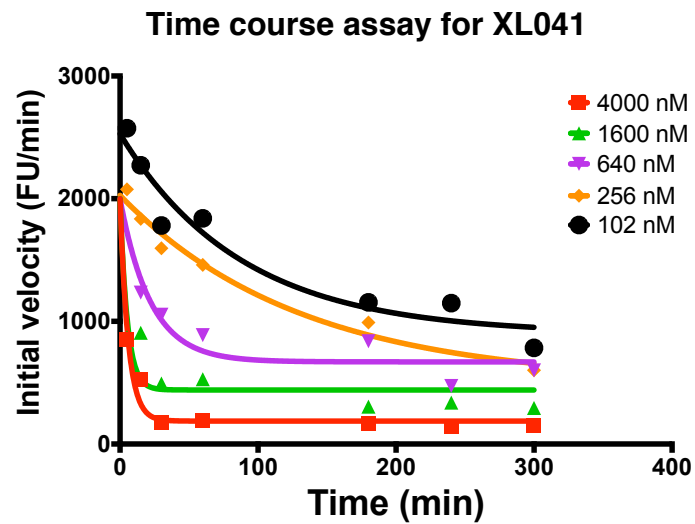
Figure S12: Top Panel: The indicated Ewing Sarcoma cell lines were treated with XL177B or GNE-6640 for 3 days, and viability was assessed by Cell Titer Glo. Red lines indicate p53-WT cells, and black lines indicate p53-mutant cells. Bottom Panel: TC32 cells stably expressing the indicated sgRNA was treated for 3 days with XL177B or GNE-6640 at the indicated doses, and viability was assessed by Cell Titer Glo.

Figure S13: A 1:1 mixture of A549 (top panel) or RKO (bottom panel) cells expressing FF and Renilla-sgTP53 were treated with sgRNAs targeting the indicated genes, then allowed to grow for 17 days, with luminescence readings taken at the indicated timepoints. WT:KO ratio is equivalent to the FF:Renilla ratio.

Figure S14: Original Western blots of figures in main text.

Figure S15: The general synthetic route used to synthesize XL177A, XL177B, XL112, XL041, and XL058.

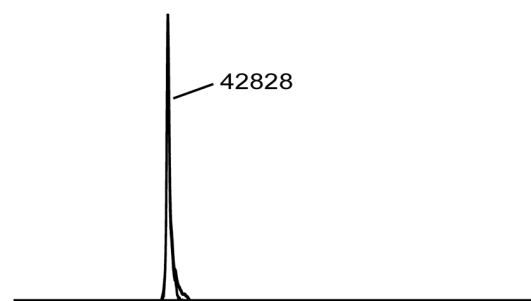
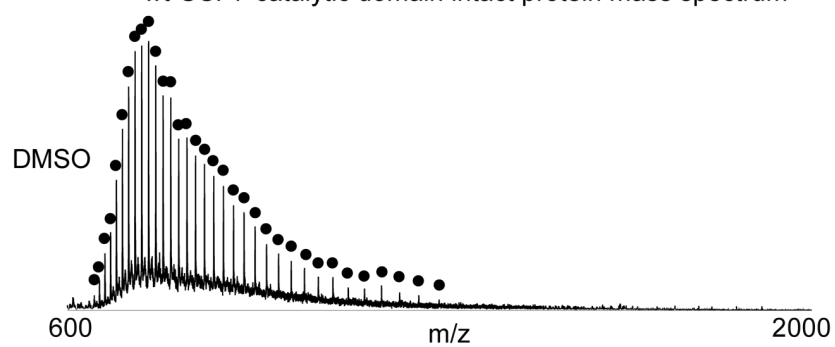
Figure S16: The synthetic route used to synthesize XL024.



K _{inact} (min^{-1})	0.2237±0.07434
K _i (μM)	2.8±1.8

Figure S1

wt-USP7 catalytic domain intact protein mass spectrum



No Labeling

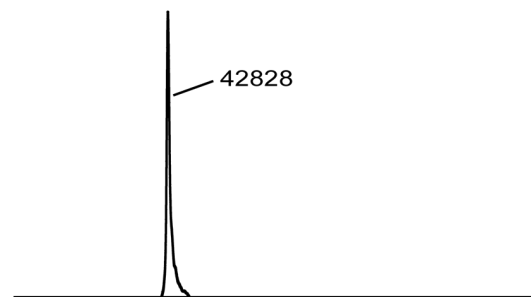
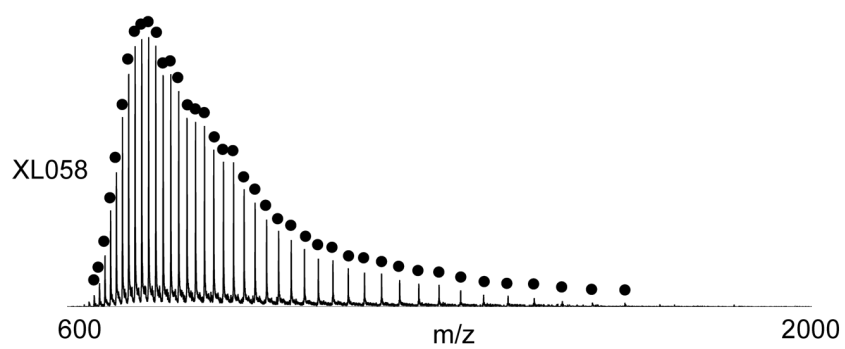


Figure S2

C223A USP7 catalytic domain intact protein mass spectrum

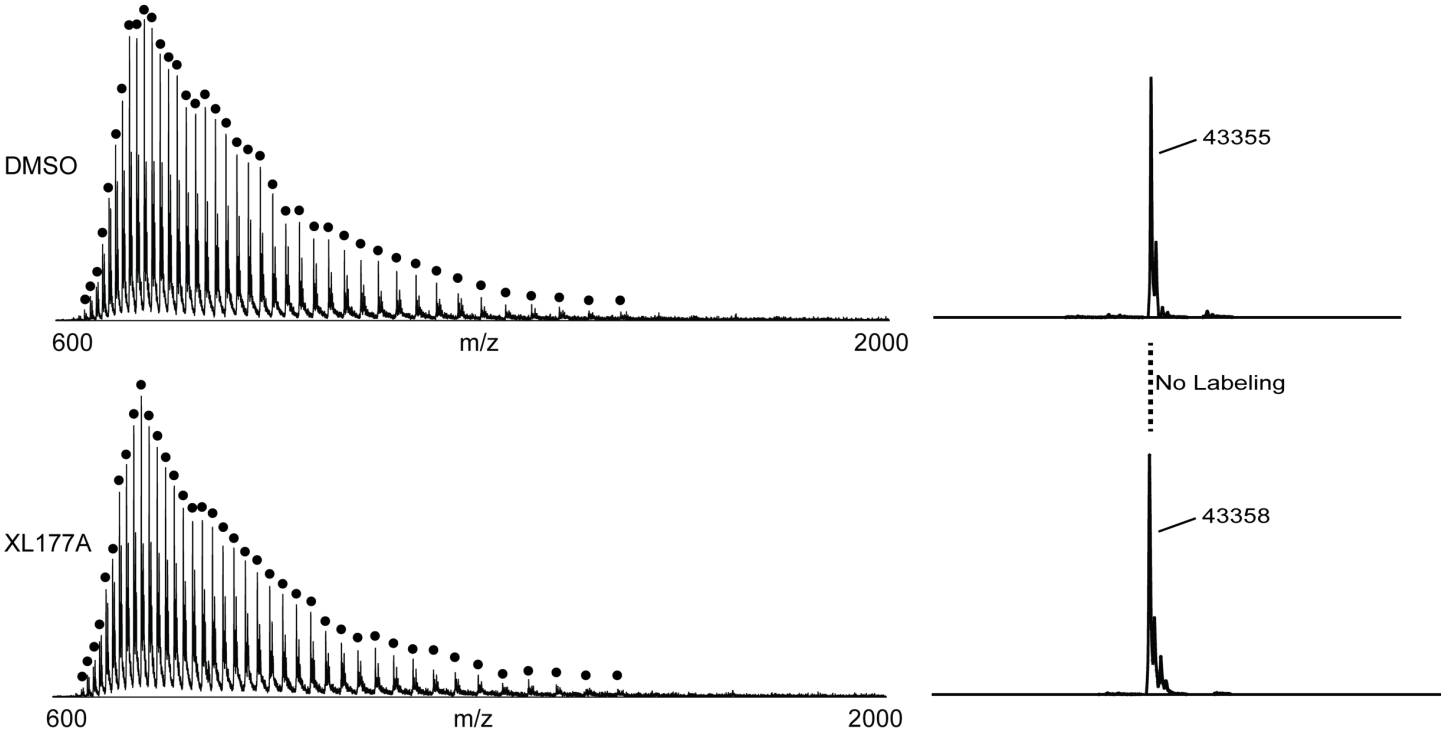
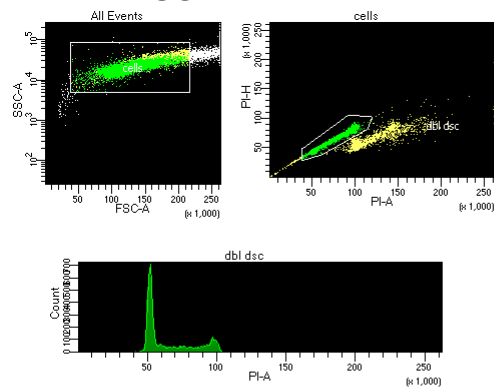
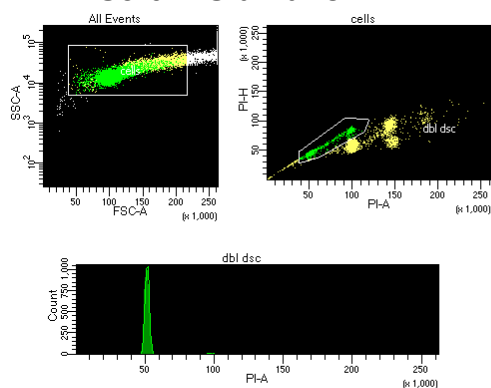


Figure S3

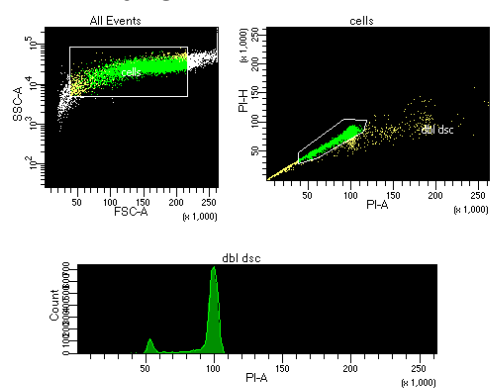
DMSO



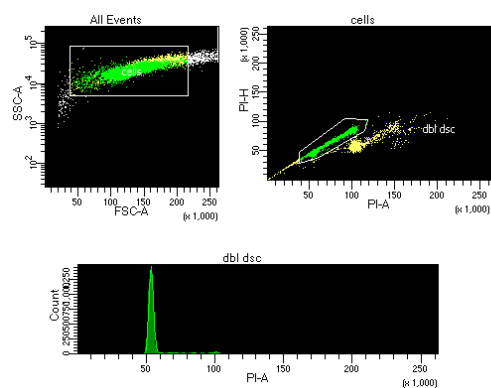
Serum Starvation



Taxol



XL177A



XL177B

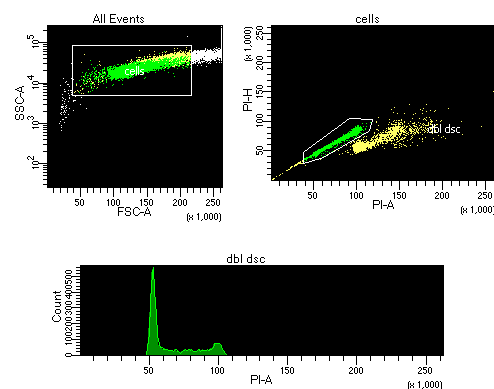


Figure S4

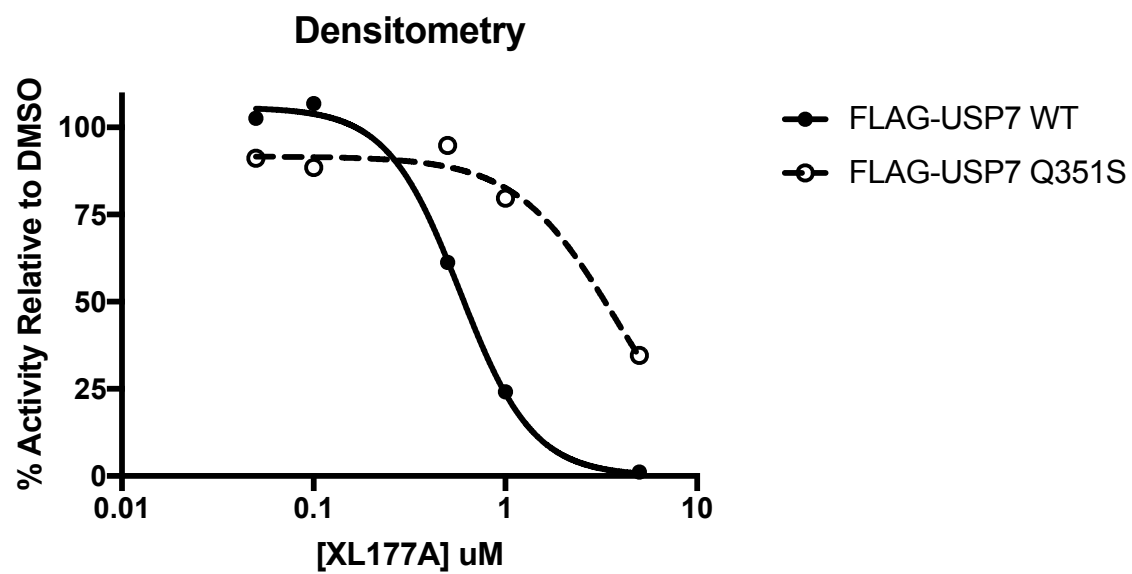
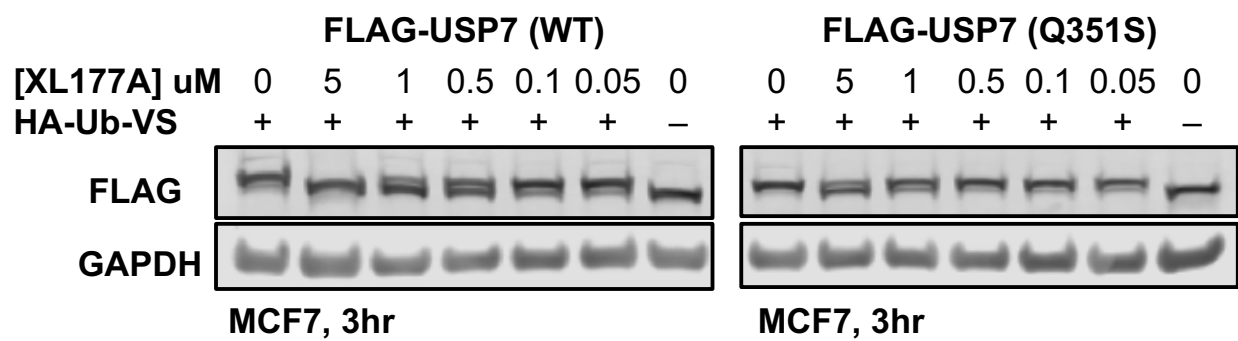
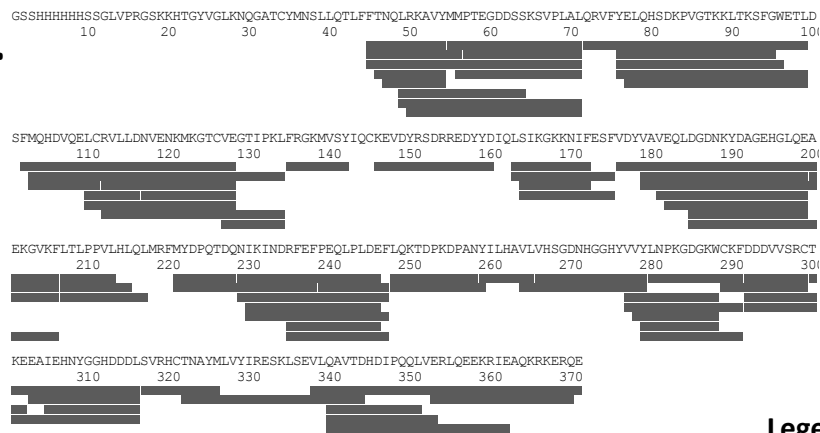


Figure S5

A.

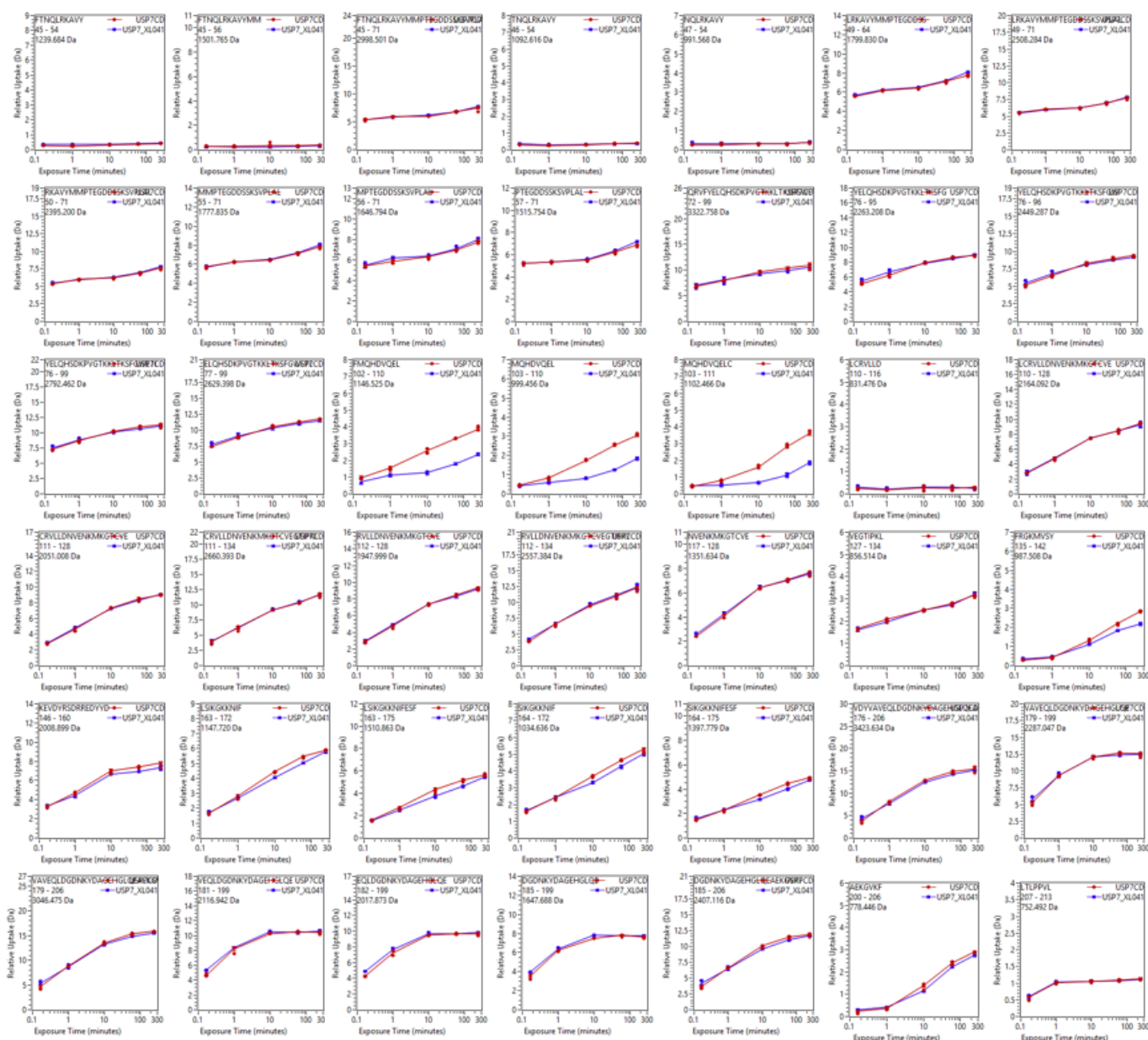


Legend

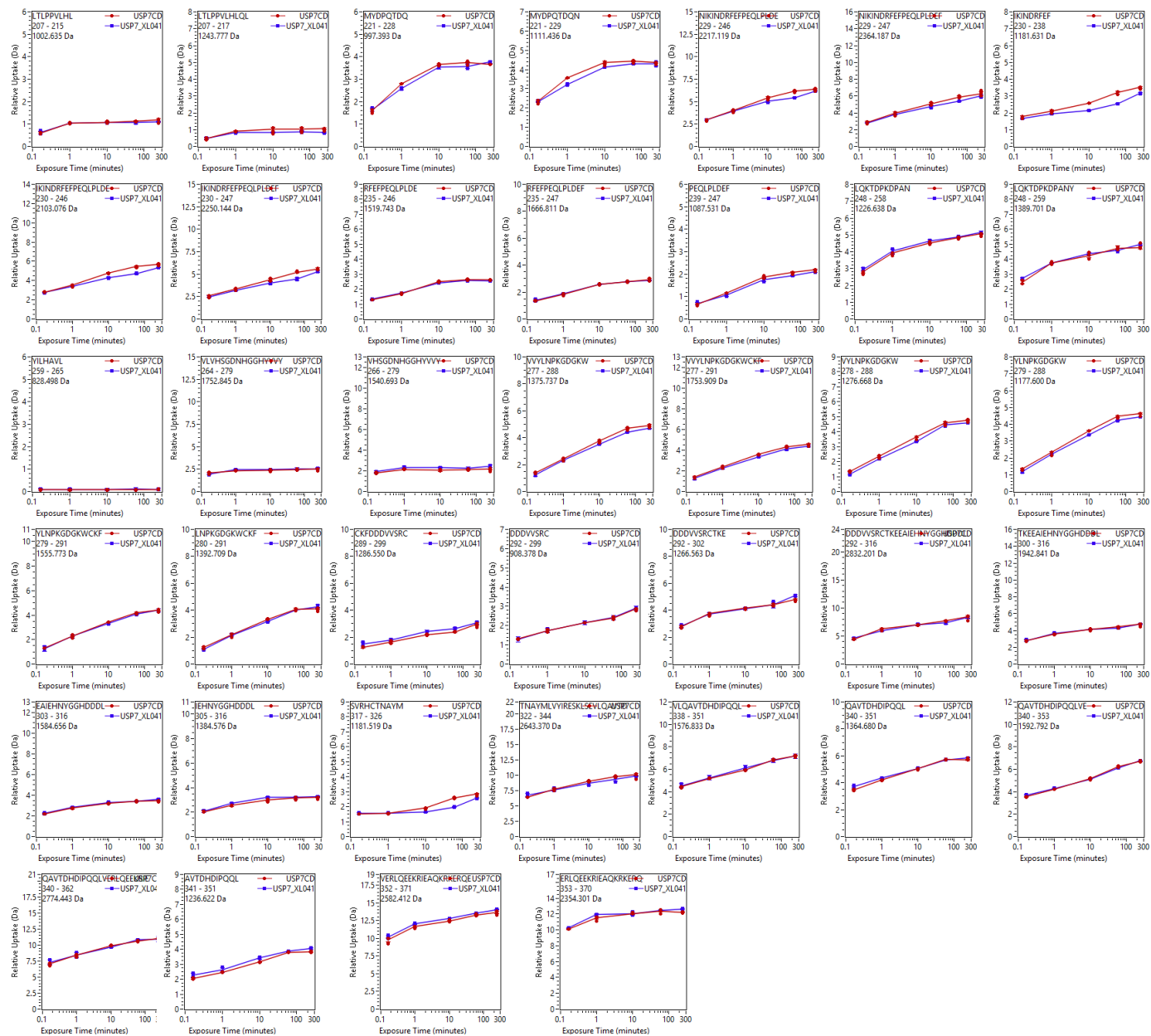
● USP7

● USP7+ XL041

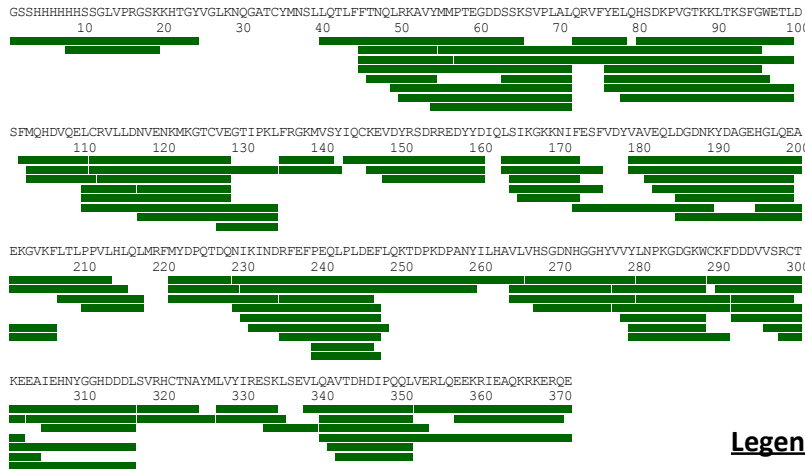
Total: 81 Peptides, 85.4% Coverage, 3.81 Redundancy



A. continued

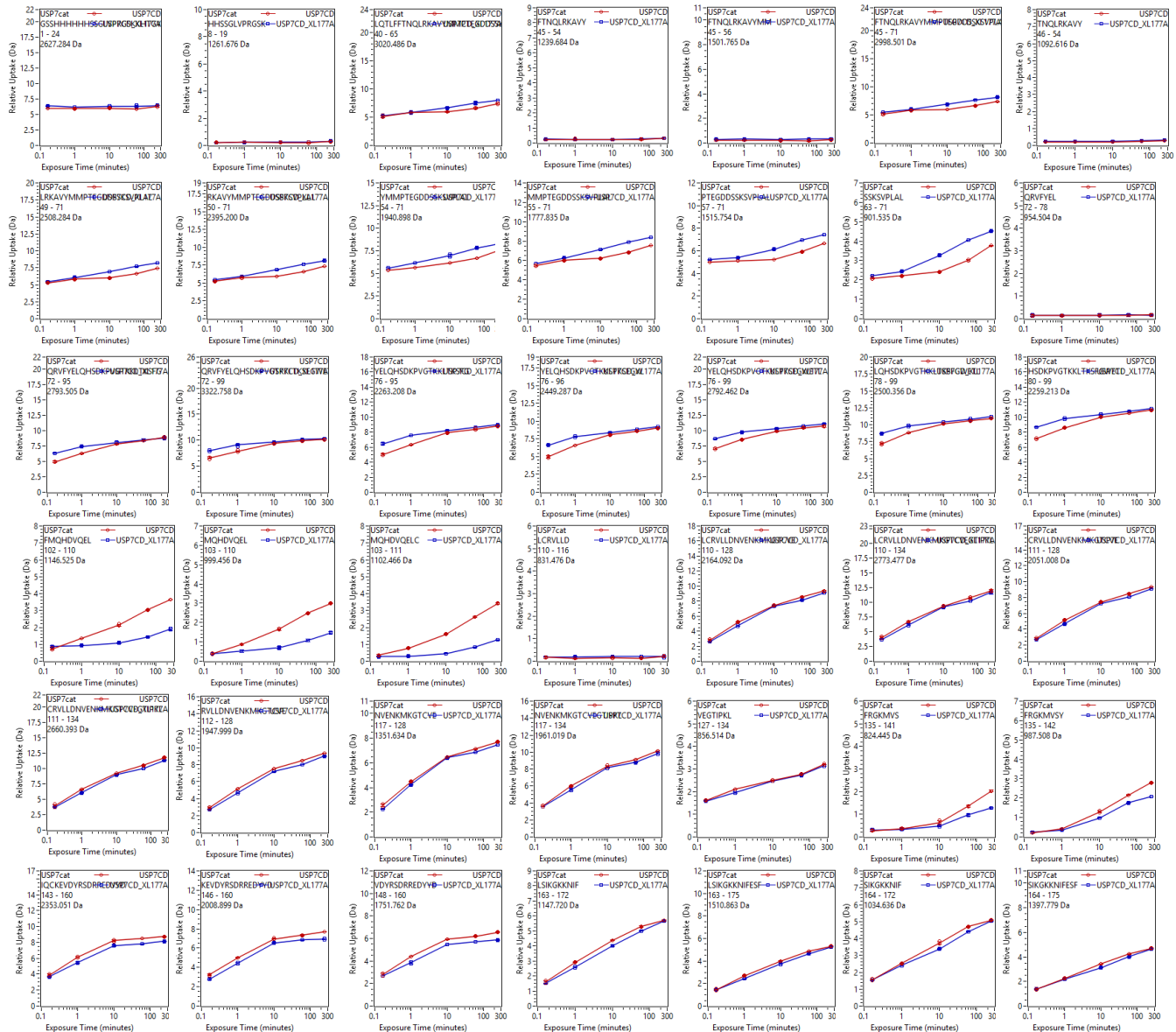
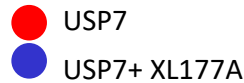


B.

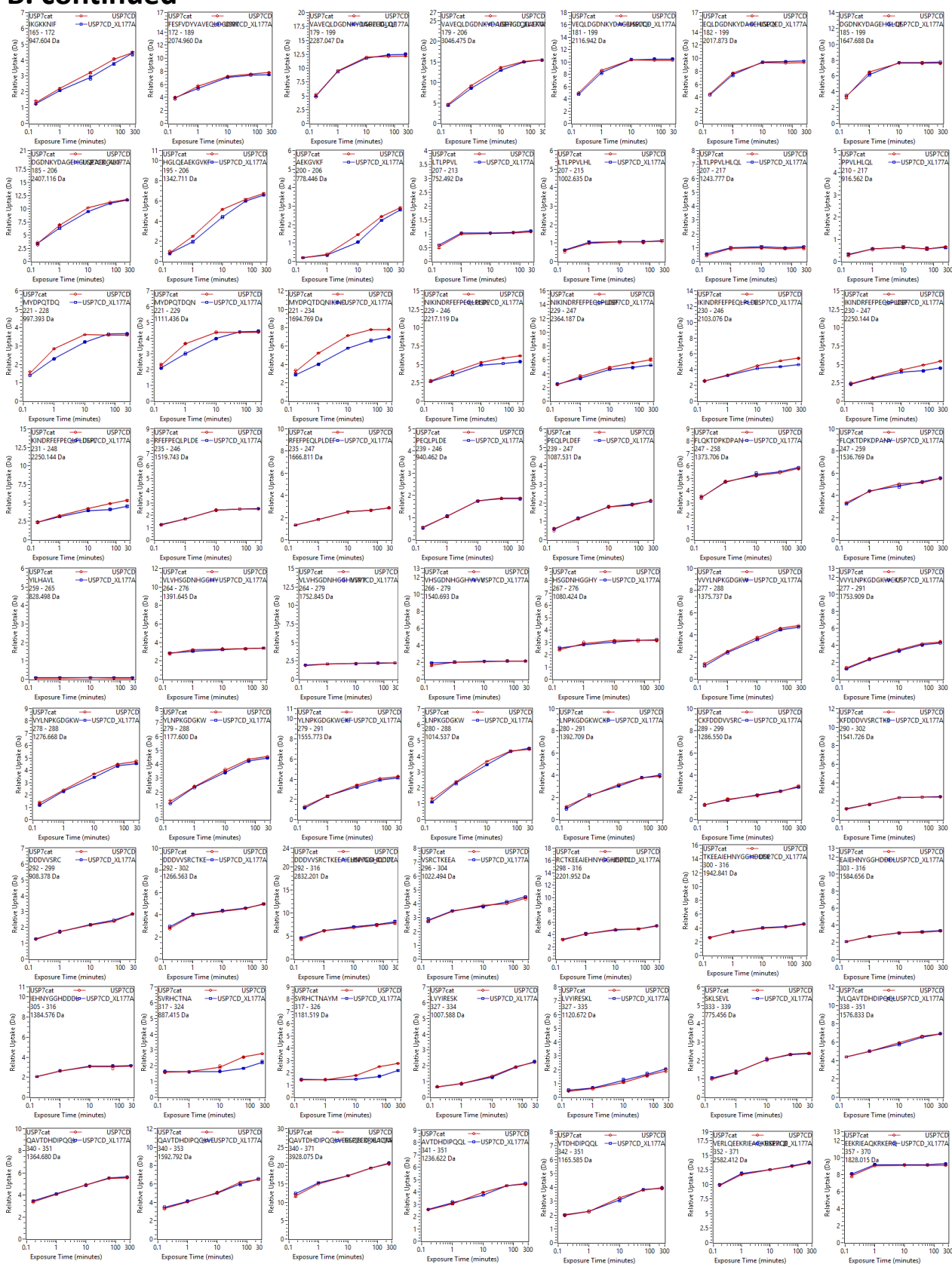


Total: 105 Peptides, 94.1% Coverage, 4.33 Redundancy

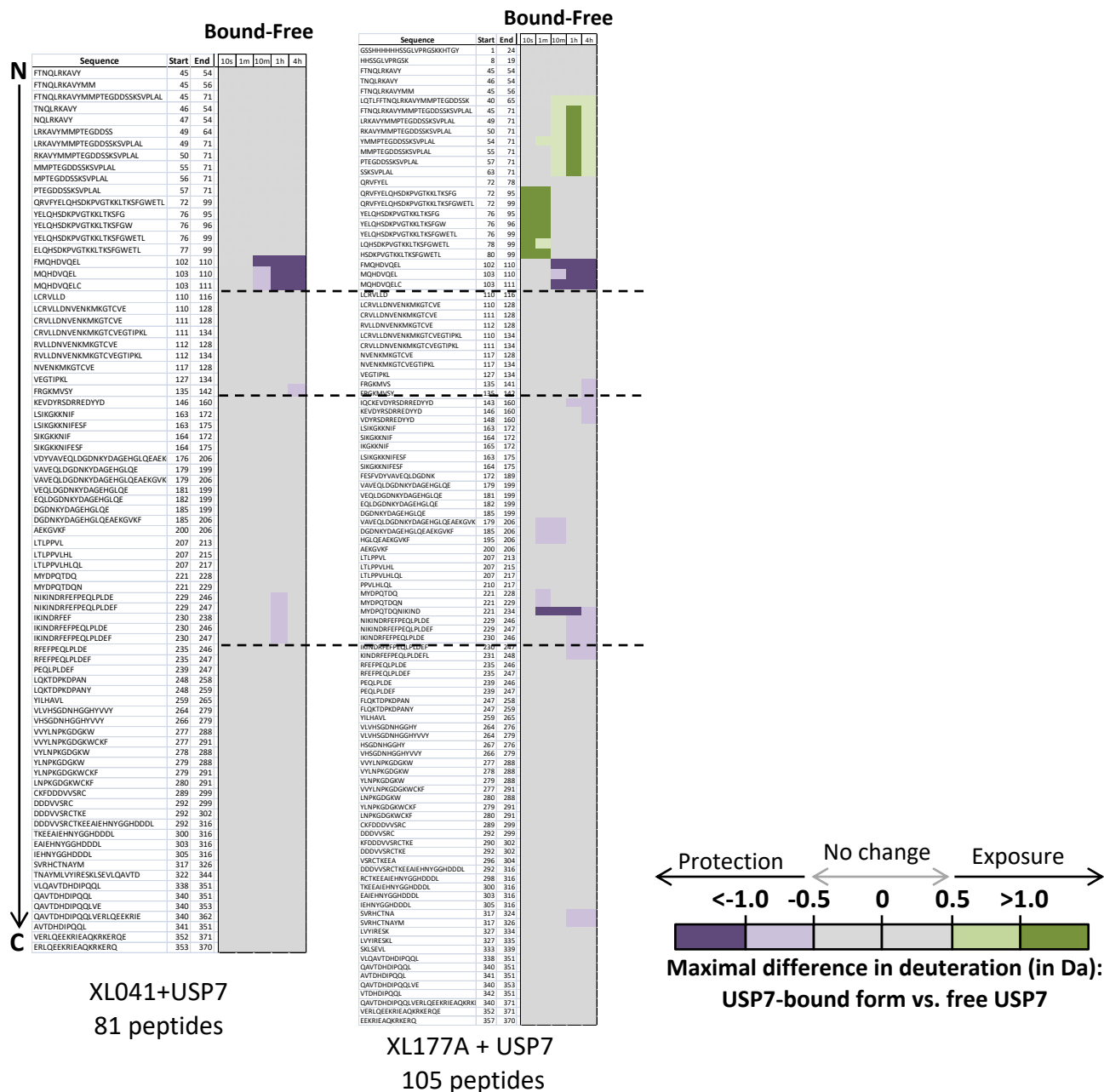
Legend



B. continued



C.



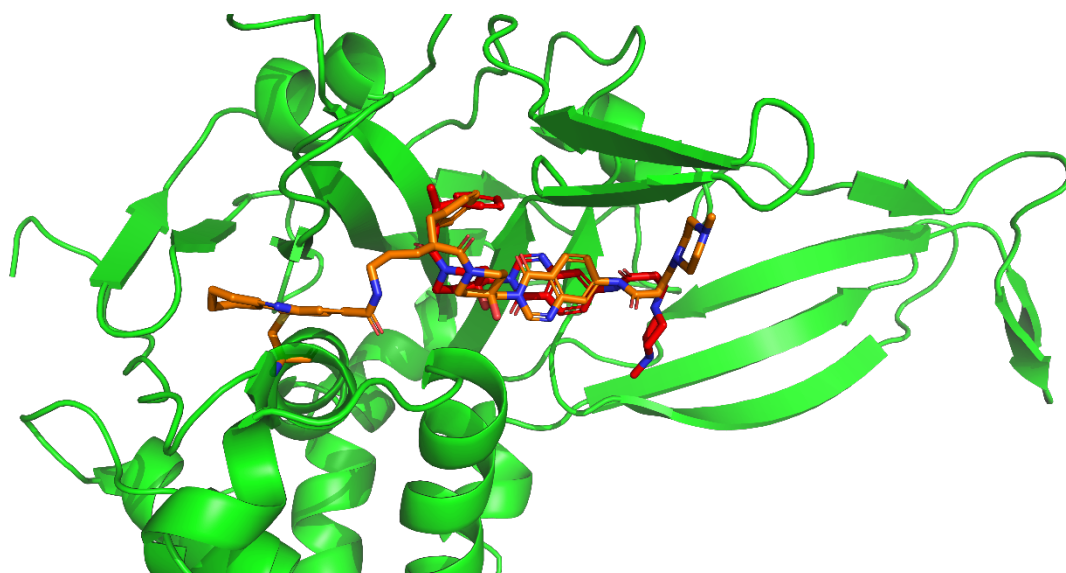


Figure S7



Figure S8

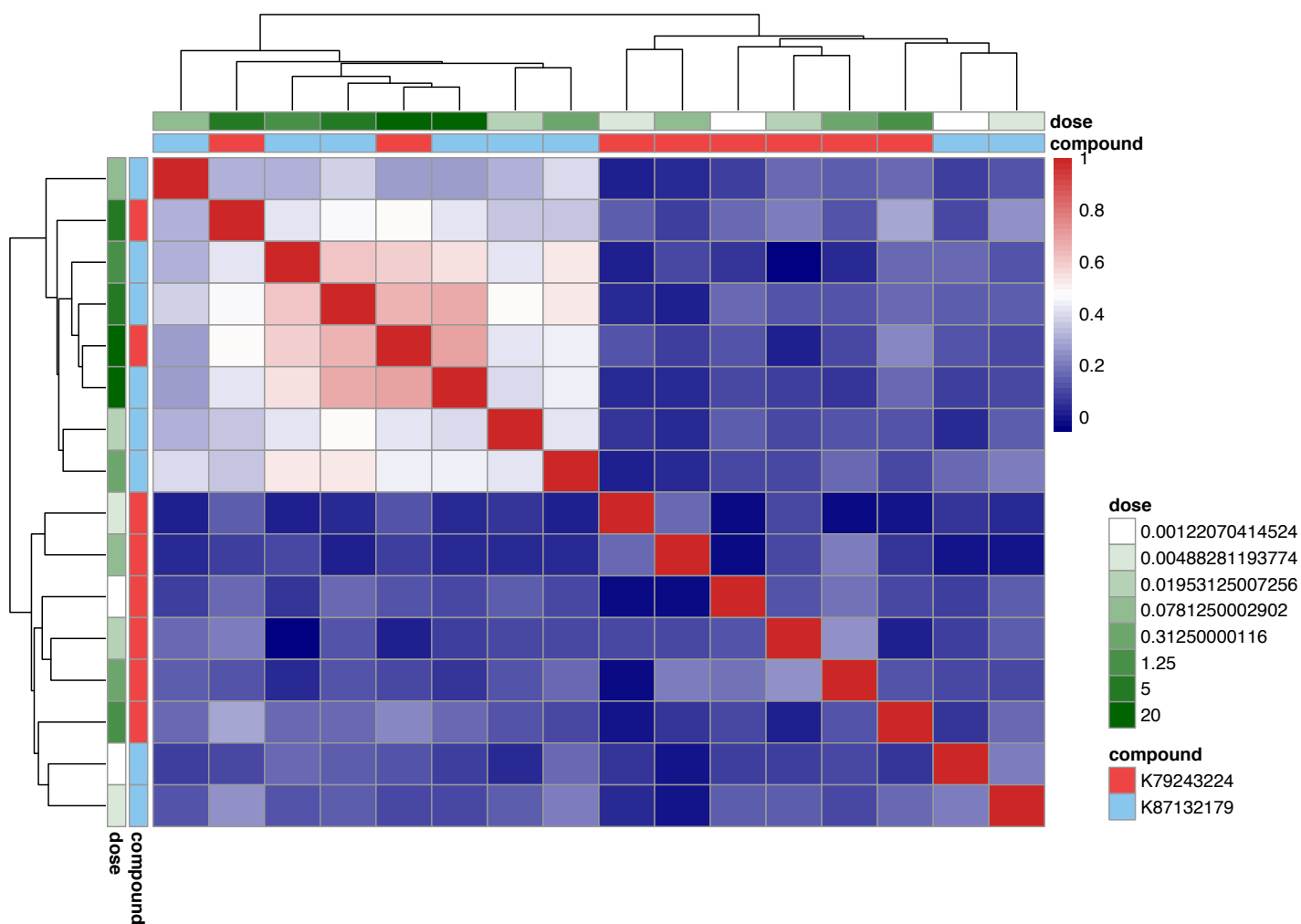
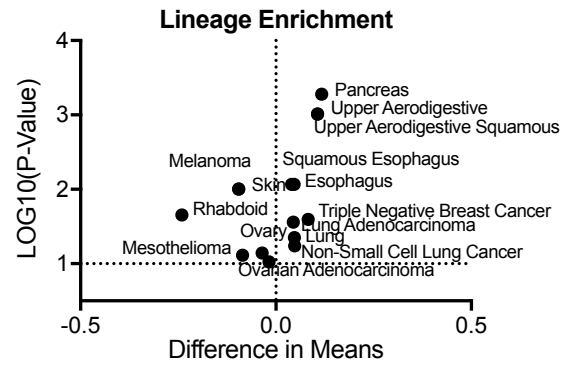
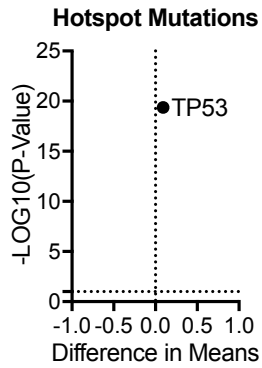
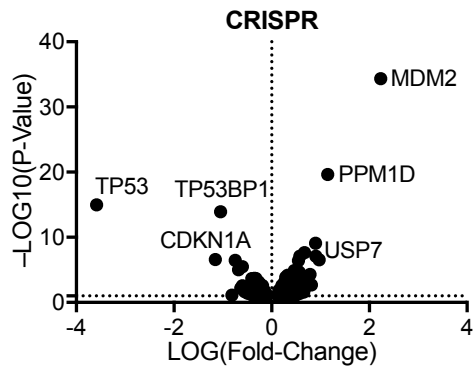


Figure S9

Nutlin-3A



XL177B

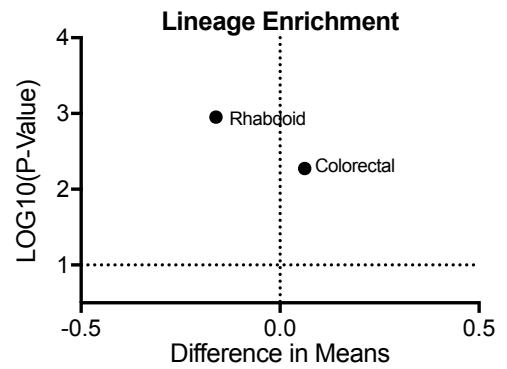
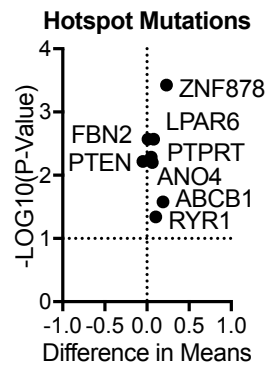
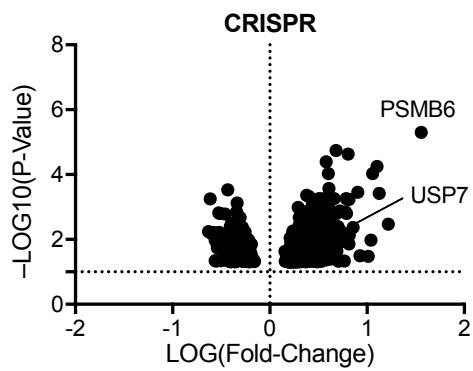


Figure S10

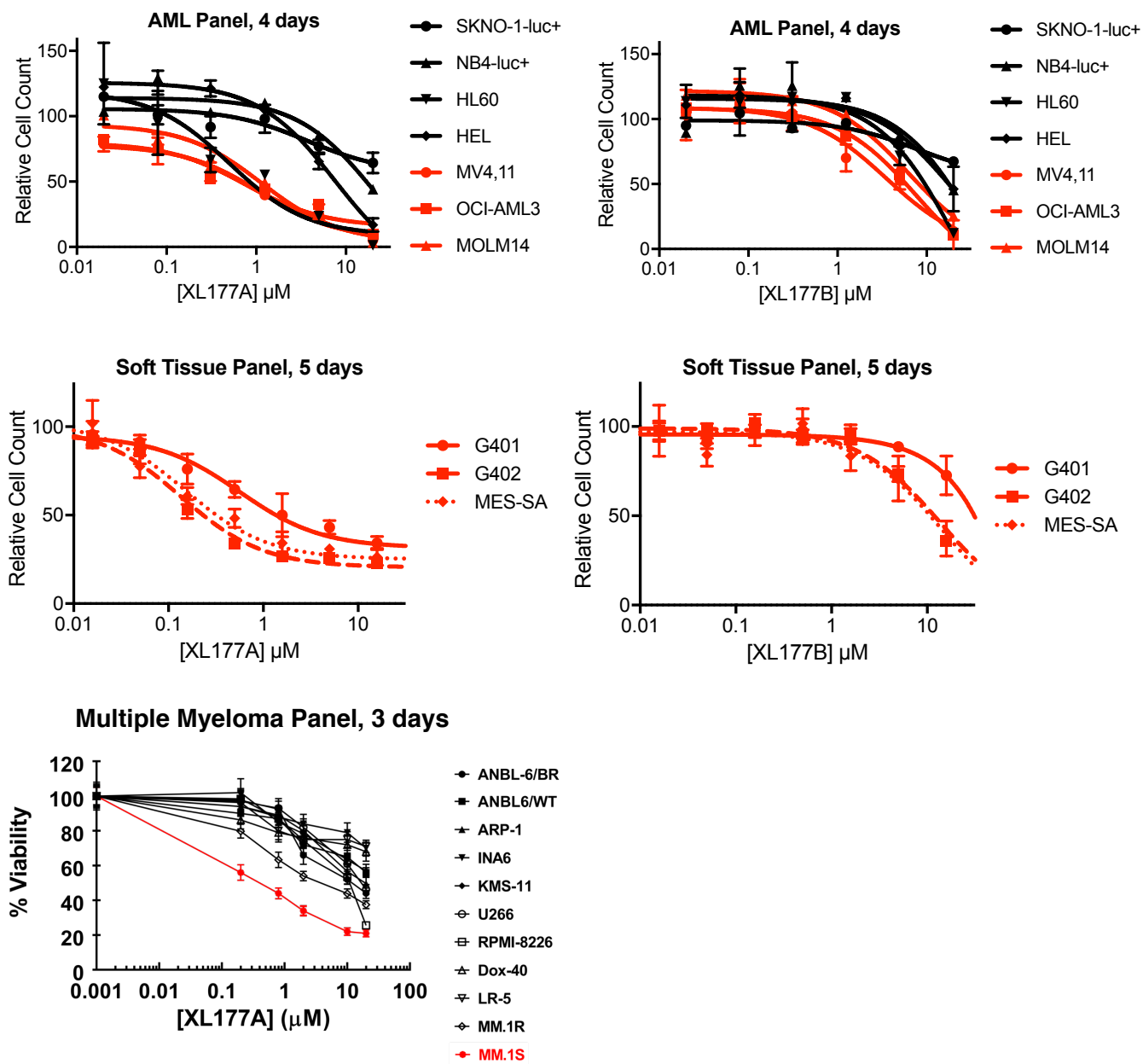
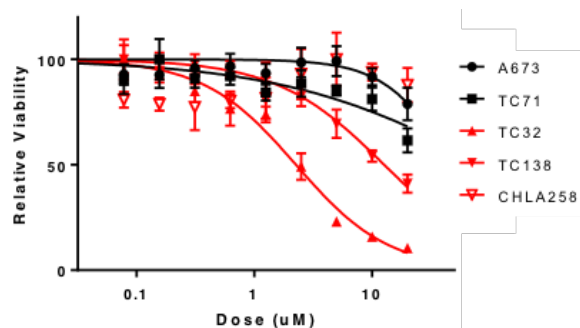
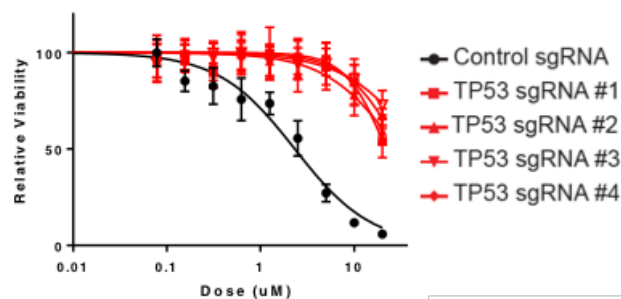


Figure S11

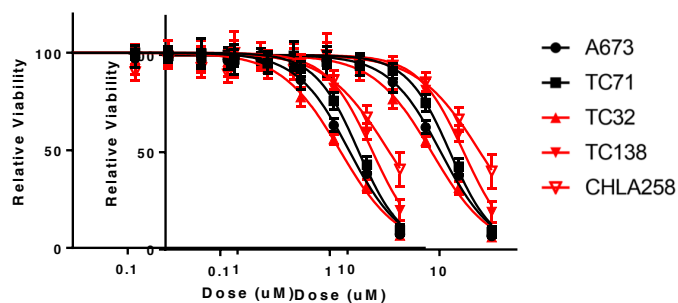
XL177B, 3 day proliferation



XL177B, TC32 cells, 3 days



SB1 GNE-6640, 3 day proliferation
Genentech compound



SB1 GNE-6640, 3 day proliferation, TC32 Cells
Genentech compound

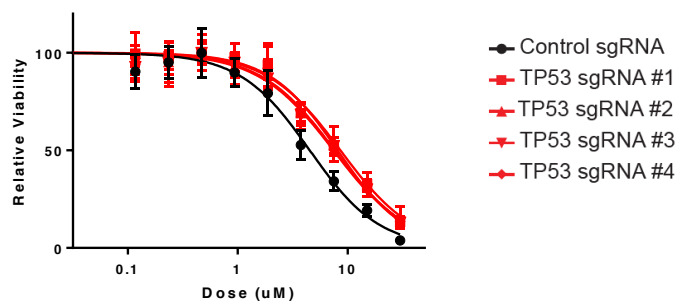
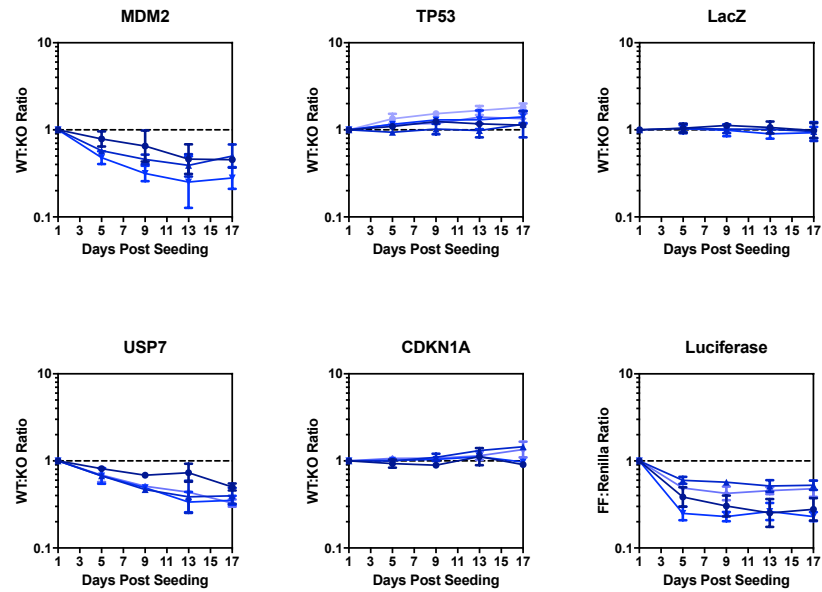


Figure S12

A549



RKO

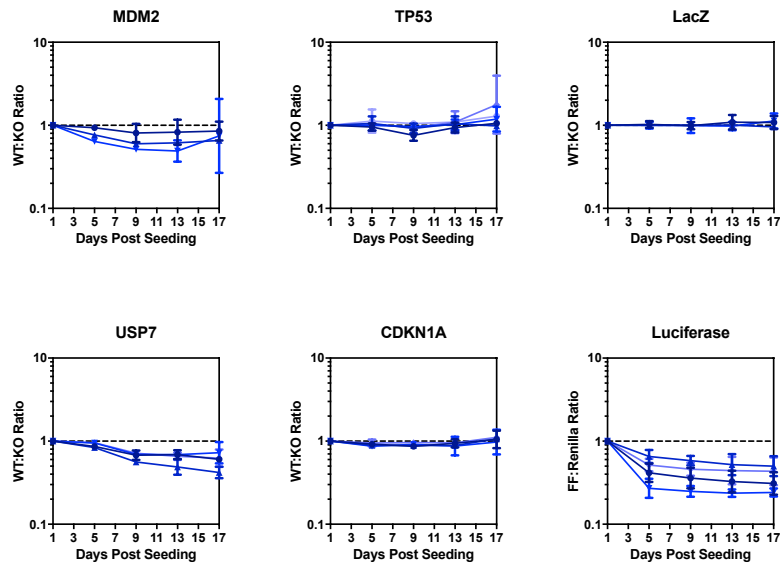
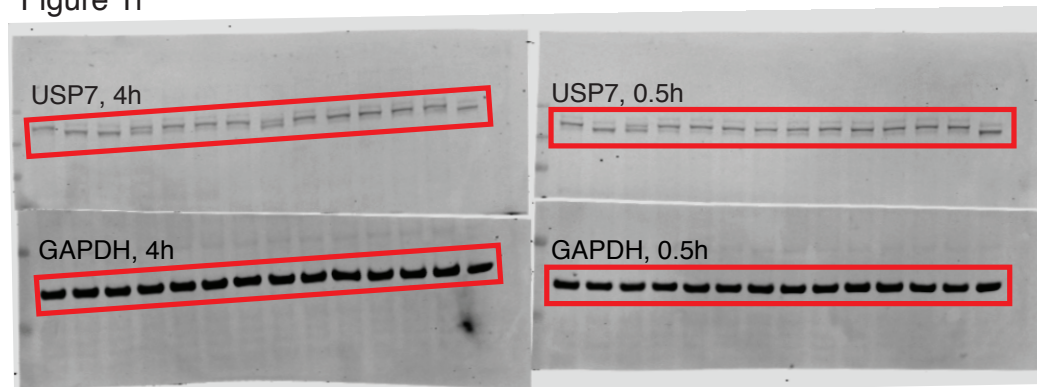


Figure S13

Figure 1f



Figures 2a and 2c (partial)

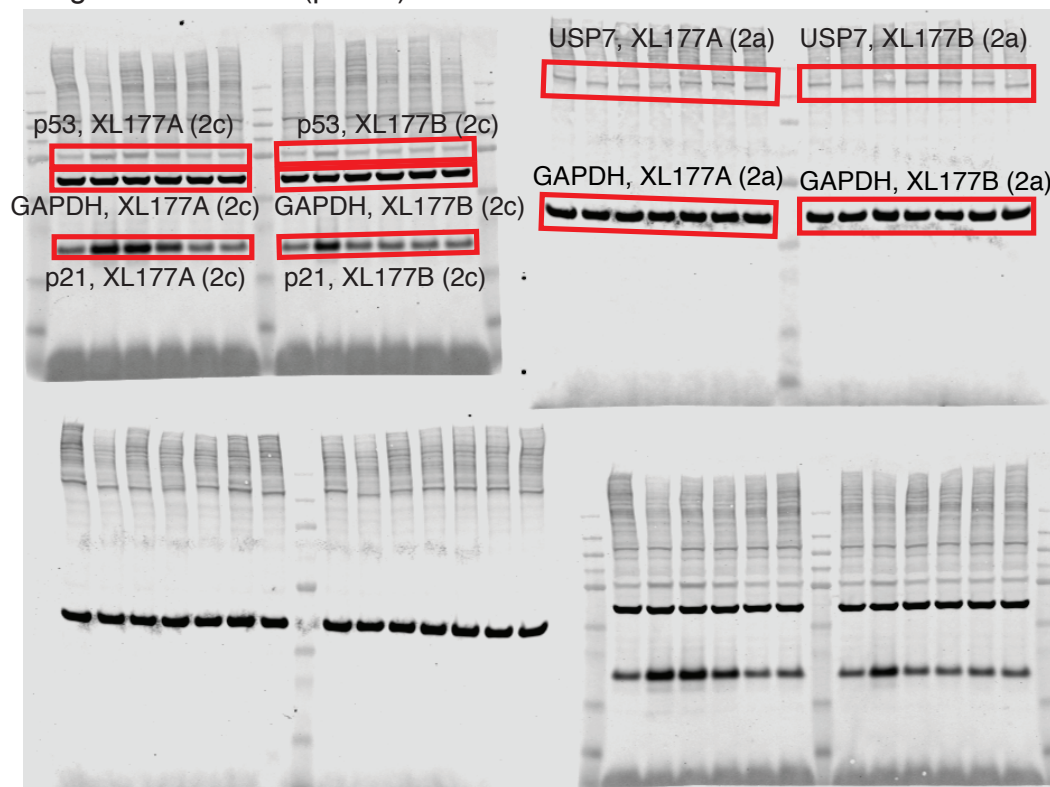


Figure S5

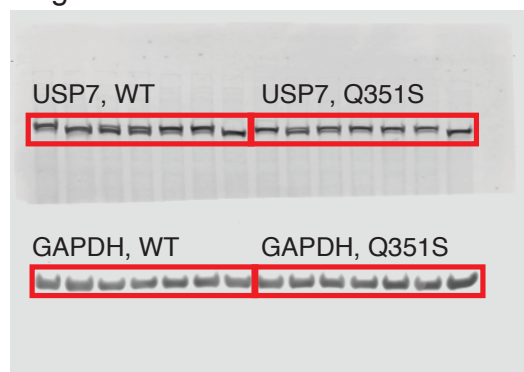


Figure 2c (partial)

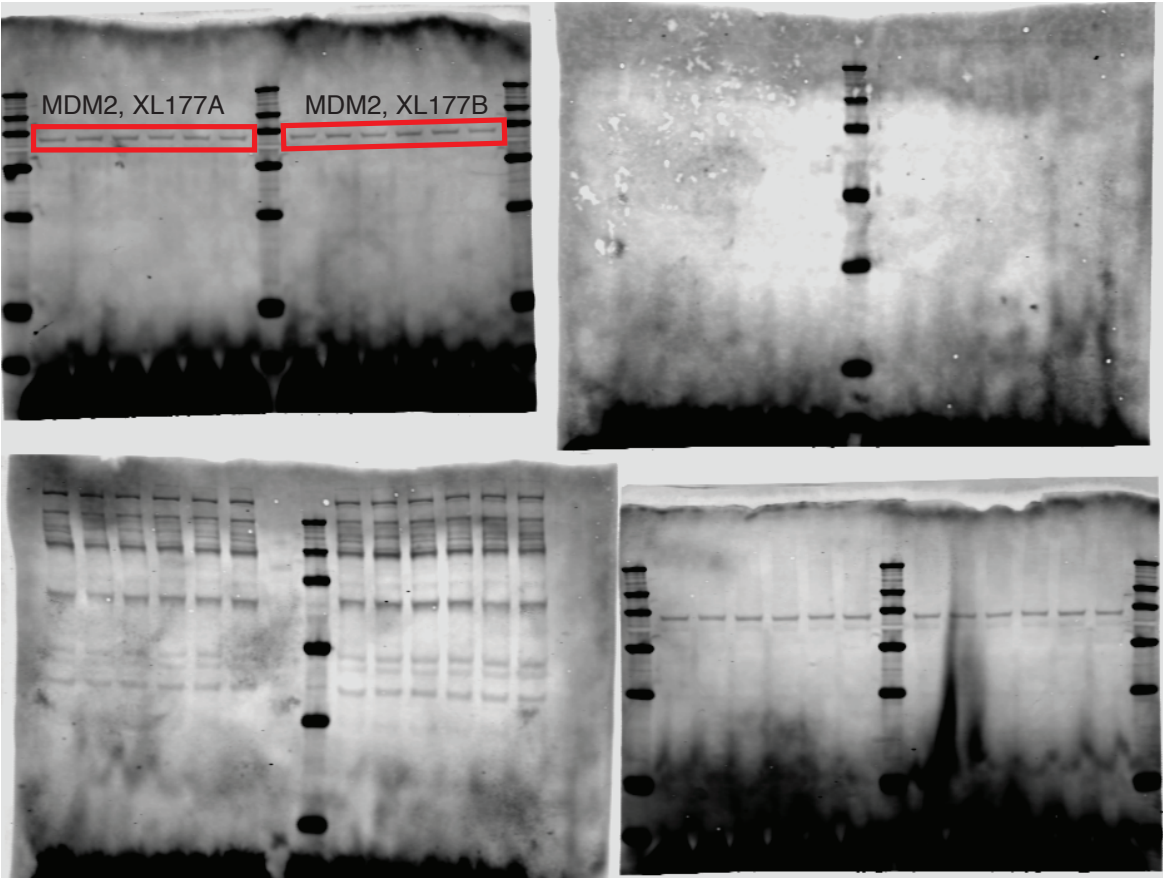


Figure 2b

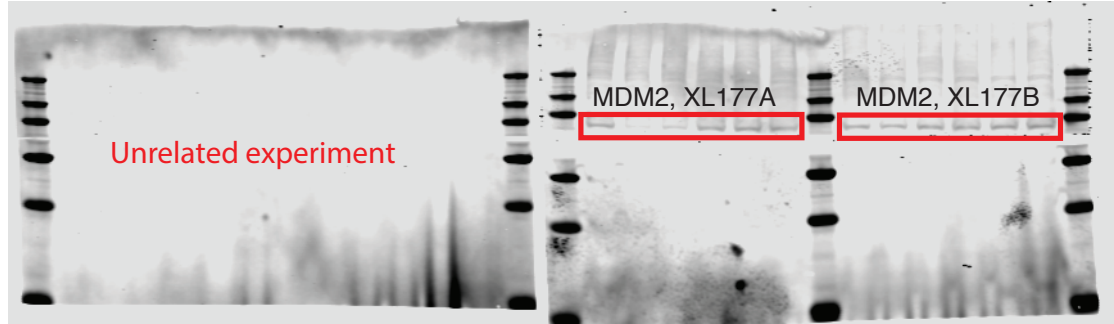


Figure 2b

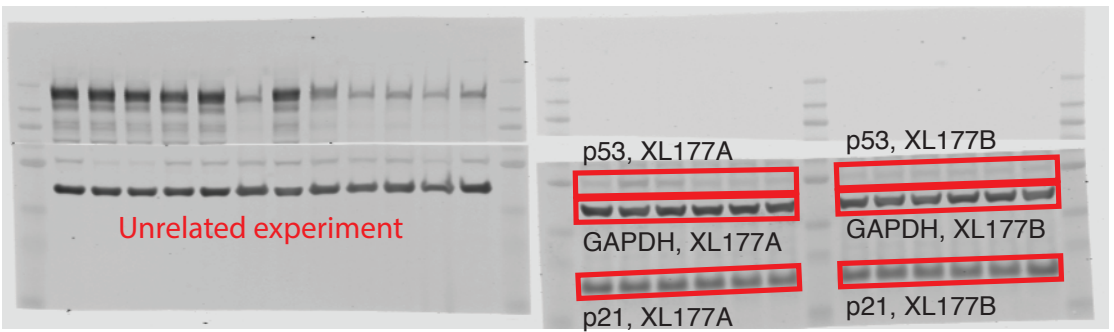
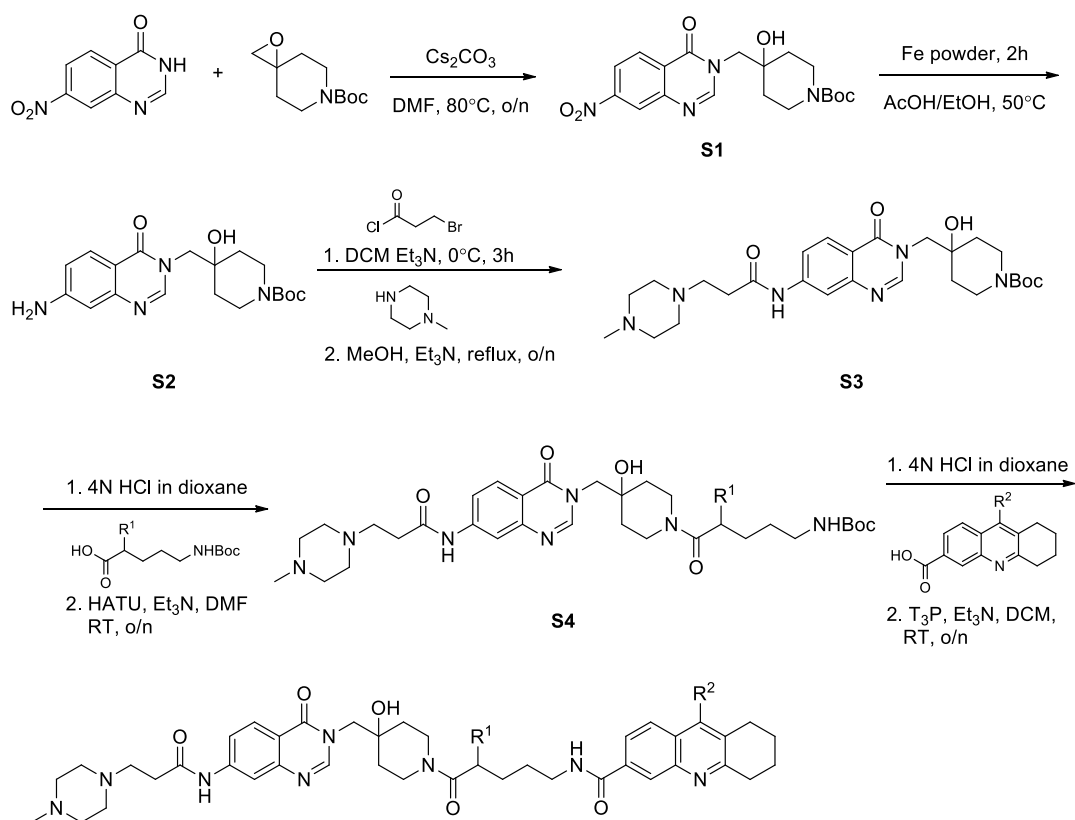


Figure S14



	XL177A	XL177B	XL112	XL041	XL058
R ¹	(<i>S</i>)-Bn	(<i>R</i>)-Bn	(<i>rac</i>)-Bn	H	(<i>rac</i>)-Bn
R ²	Cl	Cl	Cl	Cl	H

Figure S15

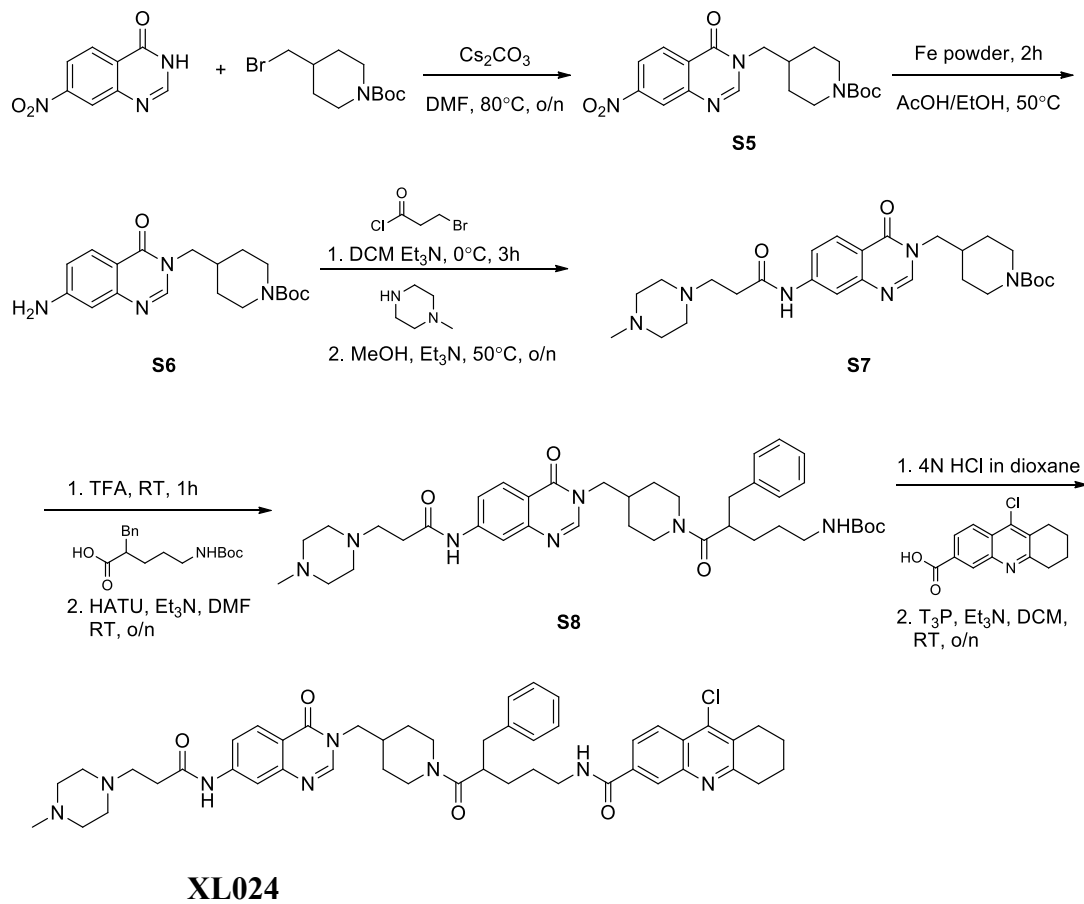


Figure S16

Supplementary Synthetic Methods

Synthesis of XL177A, XL177B, XL112, XL041, and XL058 (see Figure S15):

Step 1 (Synthesis of **S1**): 7-nitroquinazolin-4(3*H*)-one (1.55g, 8.1mmol) and *tert*-butyl-1-oxa-6-azaspiro[2.5]octane-6-carboxylate (1.90g, 8.9mmol) were added into 20mL DMF. Cesium carbonate (7.82g, 24.0mmol) was added in one portion. The mixture was heated at 80°C overnight. The mixture was diluted with EtOAc, then washed with sat. NaCl. Combined organic layer was concentrated under reduced pressure. The crude product was purified by flash chromatography (EtOAc: hexanes: 50%-70%) to afford 2.42g **S1** (75%). ¹H NMR (500 MHz, CDCl₃) δ 8.54 (d, *J* = 2.1 Hz, 1H), 8.44 (t, *J* = 8.6 Hz, 1H), 8.26 (dd, *J* = 8.8, 2.2 Hz, 1H), 8.20 (s, 1H), 4.10 (s, 2H), 3.88 (s, 2H), 3.14 (t, *J* = 11.6 Hz, 2H), 1.73 – 1.48 (m, 5H), 1.44 (s, 9H). LCMS (ESI) *m/z* 304.97 (M+H–Boc) [(M+H)⁺ C₁₉H₂₅N₄O₆⁺ calcd for 405.18]

Step 2 (Synthesis of **S2**): Compound **S1** (2.4g, 6.0mmol) was suspended in 20mL solvent (EtOH/AcOH=1:1). 4 eq. of Fe powder was added in portions. The mixture was stirred for 1 hour at 55°C. Then the reaction was cooled down to room temperature, and filtered through a pad of Celite. The filtrate was concentrated under reduced pressure to afford the crude product, which was then purified by flash chromatography (10% MeOH in EtOAc) to afford 2.1g product **S2** (93%) ¹H NMR (500 MHz, DMSO) δ 8.04 (s, 1H), 7.79 (d, *J* = 8.7 Hz, 1H), 6.72 (dd, *J* = 8.7, 1.9 Hz, 1H), 6.61 (d, *J* = 2.0 Hz, 1H), 6.09 (s, 2H), 4.87 (s, 1H), 3.89 (s, 2H), 3.64 (d, *J* = 12.0 Hz, 2H), 3.05 (s, 2H), 1.54 – 1.24 (m, 13H). LCMS (ESI) *m/z* 374.97 [(M+H)⁺ C₁₉H₂₇N₄O₄⁺ calcd for 375.20]

Step 3 (Synthesis of **S3**): Compound **S2** (2.1g, 5.6mmol) was dissolved in anhydrous 10mL dichloromethane under N₂ at 0°C. 3.0 eq. of Et₃N was added. Then 3-bromopropionyl chloride (1.15g, 6.7mmol) was added dropwise. The mixture was stirred at 0°C for 1 hour, then quenched with MeOH, and concentrated under reduced pressure. The solid residue was directly used for the following step without further purification. The crude product from last step was dissolved in 10mL MeOH, then 3.0eq of Et₃N was added. Into the stirred mixture was added 1-methylpiperazine (0.67g, 6.7mmol) dropwise. After the addition completed, the mixture was stirred for 1 hour at 50°C. Then the reaction mixture was cooled down to room temperature and concentrated under reduced pressure, then directly subjected to HPLC purification (MeOH/H₂O with 4% TFA) to afford 2.1g product **S3** (73% in two steps) ¹H NMR (500 MHz, MeOD) δ 8.28 (s, 1H), 8.20 (d, *J* = 8.8 Hz, 1H), 8.12 (d, *J* = 1.9 Hz, 1H), 7.69 (dd, *J* = 8.7, 2.0 Hz, 1H), 4.11 (s, 2H), 3.82 (d, *J* = 13.4 Hz, 2H), 3.23 (m, 2H), 2.85 (t, *J* = 7.0 Hz, 2H), 2.79 – 2.50 (m, 10H), 2.37 (s, 3H), 1.72 – 1.62 (m, 2H), 1.50 (d, *J* = 17.4 Hz, 11H). LCMS (ESI) *m/z* 529.08 [(M+H)⁺ C₂₇H₄₁N₆O₅⁺ calcd for 529.31].

Step 4 (Synthesis of **S4** (R¹=(*S*)-Bn, R²=Cl)): **S3** (0.53g, 1.0mmol) was dissolved in 3mL DCM, then 5mL 4M HCl in 1,4-dioxane was added in portions. The solution was stirred for 1 hour at room temperature. Then the

mixture was concentrated under reduced pressure, and left on high vacuum overnight to remove residual acid. Then the product (0.11g, 0.25mmol) was dissolved in 3mL DMF, and basified by adding 10 eq of Et₃N. Sequentially, (*S*)-2-benzyl-5-((tert-butoxycarbonyl)amino)pentanoic acid (0.11g, 0.35mmol) and HATU (0.16g, 0.4mmol) were added to the solution. The resultant solution was stirred overnight. Then the mixture was directly subjected to HPLC purification (MeOH/H₂O with 4% TFA) to afford 183mg **S4** (quantitative) ¹H NMR (500 MHz, DMSO) δ 10.51 (s, 1H), 8.16-8.04 (m, 2H, conformer), 8.01 (dd, *J* = 5.0, 1.9 Hz, 1H), 7.61 (ddd, *J* = 9.8, 8.3, 2.0 Hz, 1H), 7.29 – 7.19 (m, 2H), 7.19 – 7.07 (m, 3H), 6.76 (m, 1H), 4.84 (s, 1H), 4.11 (m, 1H), 4.05 – 3.87 (m, 2H), 3.80 (d, *J* = 13.7 Hz, 1H), 3.67 – 3.49 (m, 3H), 3.16 – 3.03 (m, 2H), 2.98 (s, 1H), 2.85 (m, 3H), 2.77 – 2.58 (m, 5H), 2.55 (m, 3H), 2.22 (s, 3H), 1.61 – 1.44 (m, 1H), 1.43 – 1.33 (m, 9H, conformer), 1.33 – 1.22 (m, 3H), 1.22 – 1.02 (m, 4H), 0.39 (m, 1H). LCMS (ESI) *m/z* 718.00 [(*M*+*H*)⁺ 718.43 calcd for C₃₉H₅₆N₇O₆⁺]

Step 5 (Synthesis of **XL177A**): **S4** (0.18g, 0.25mmol) was dissolved in 4M HCl in 1,4-dioxane, and stirred for 1h at room temperature. Then the mixture was concentrated under reduced pressure, and left on high vacuum overnight to remove residual solvent. Then the product (0.16g, 0.25mmol) was dissolved in 5mL DCM with 10 eq. of Et₃N. Into the solution were added 9-chloro-5,6,7,8-tetrahydroacridine-3-carboxylic acid (0.10g, 0.4mmol) and T3P (50% in EtOAc) (0.42g, 1.3mmol). The solution was stirred at room temperature under nitrogen overnight. Then the mixture was concentrated under reduced pressure, and purified sequentially by flash chromatography (MeOH in EtOAc: 0%-70%) and HPLC (MeOH/H₂O with 4% TFA) to afford 98mg **XL177A** (46%).

XL177A

(*S*)-*N*-(4-benzyl-5-(4-hydroxy-4-((7-(3-(4-methylpiperazin-1-yl)propanamido)-4-oxoquinazolin-3(4*H*)-yl)methyl)piperidin-1-yl)-5-oxopentyl)-9-chloro-5,6,7,8-tetrahydroacridine-3-carboxamide. ¹H NMR (500 MHz, DMSO) δ 10.50 (d, *J* = 5.5 Hz, 1H), 8.85 – 8.69 (m, 1H), 8.46 (d, *J* = 14.0 Hz, 1H), 8.22 – 7.93 (m, 5H), 7.61 (dd, *J* = 16.4, 9.3 Hz, 1H), 7.17 (ddt, *J* = 32.3, 19.2, 7.4 Hz, 5H), 4.82 (d, *J* = 4.5 Hz, 1H), 4.20 – 3.93 (m, 1H), 3.93 – 3.74 (m, 2H), 3.62 (m, 2H), 3.30 (m, 4H, overlapped with H₂O), 3.20 – 3.09 (m, 2H), 3.05 (m, 2H), 2.96 (m, 2H), 2.85 (m, 1H), 2.80 – 2.70 (m, 2H), 2.66 (m, 4H), 2.58 – 2.51 (m, 3H), 2.17 (s, 4H), 1.88 (d, *J* = 3.2 Hz, 4H), 1.71 – 1.35 (m, 5H), 1.34 – 1.02 (m, 4H), 0.41 (t, *J* = 10.5 Hz, 1H). ¹³C NMR (126 MHz, DMSO) δ 172.24/172.19 (conformer), 170.98, 165.30/165.25 (conformer), 160.38/160.33 (conformer), 160.08/160.01(conformer), 149.32/149.27(conformer), 148.95, 145.59/145.56(conformer), 144.24, 139.97, 139.81/139.75(conformer), 135.19/135.17(conformer), 130.07/130.02(conformer), 128.93/128.79(conformer), 128.12/128.00(conformer), 127.43/127.37(conformer), 127.26/127.20(conformer), 125.99/125.94(conformer), 125.78, 125.37/125.32(conformer), 123.39, 118.25/118.20(conformer) 116.56/116.45(conformer), 114.67,

69.16/69.08(conformer), 54.62, 53.53/53.33(conformer), 53.44, 52.20, 45.54, 41.47/41.24(conformer), 40.90/40.68(conformer), 38.65, 37.01/36.96(conformer), 35.22, 34.63, 34.47, 34.26, 33.96, 33.56, 30.46, 29.66, 28.98, 27.05, 26.76, 21.86. LCMS (ESI) m/z 860.82 [(M+H)⁺; C₄₈H₅₈ClN₈O₅⁺ calcd for 861.42]

XL177B was synthesized according to procedure A using (*R*)-2-benzyl-5-((tert-butoxycarbonyl)amino)pentanoic acid in step 4 and 9-chloro-5,6,7,8-tetrahydroacridine-3-carboxylic acid in step 5.

(*R*)-*N*-(4-benzyl-5-(4-hydroxy-4-((7-(3-(4-methylpiperazin-1-yl)propanamido)-4-oxoquinazolin-3(4H)-yl)methyl)piperidin-1-yl)-5-oxopentyl)-9-chloro-5,6,7,8-tetrahydroacridine-3-carboxamide. ¹H NMR (500 MHz, DMSO) δ 10.49 (d, *J* = 5.6 Hz, 1H), 8.82 – 8.69 (m, 1H), 8.46 (d, *J* = 14.0 Hz, 1H), 8.20 – 7.95 (m, 5H), 7.66 – 7.55 (m, 1H), 7.30 – 7.05 (m, 5H), 4.82 (d, *J* = 4.8 Hz, 1H), 4.06 (dd, *J* = 62.9, 12.9 Hz, 1H), 3.95 – 3.75 (m, 1H), 3.61 (m, 2H), 3.31 – 3.21 (m, 4H, overlapped with H₂O), 3.12 (m, 2H), 3.04 (m, 2H), 2.95 (m, 2H), 2.83 (m, 1H), 2.79 – 2.70 (m, 2H), 2.70 – 2.60 (m, 4H), 2.53 (m, 3H), 2.20 (s, 4H), 1.94 – 1.81 (m, 4H), 1.70 – 1.34 (m, 5H), 1.34 – 1.00 (m, 4H), 0.40 (dt, *J* = 12.0, 9.1 Hz, 1H). ¹³C NMR (126 MHz, DMSO) δ 172.75/172.70 (conformer), 171.45, 165.81/165.77 (conformer), 160.89/160.84 (conformer), 160.58/160.52 (conformer), 149.82/149.78 (conformer), 149.45, 146.09/146.06 (conformer), 144.74, 140.47, 140.32/140.25 (conformer), 135.69/135.67 (conformer), 130.58/130.53 (conformer), 129.43/129.29 (conformer), 128.63/128.51 (conformer), 127.93/127.87 (conformer), 127.77/127.70 (conformer), 126.50/126.44 (conformer), 126.28, 125.87/125.82 (conformer), 123.90, 118.76, 117.07/116.96 (conformer), 115.18, 69.67/69.59 (conformer), 54.98, 54.04, 53.88, 52.52, 45.83, 41.97/41.75 (conformer), 41.41/41.19 (conformer), 39.15, 37.52/37.46 (conformer), 35.73, 35.13, 34.97, 34.74, 34.46, 34.06, 30.97, 30.16, 29.48, 27.56, 27.26, 22.36. LCMS (ESI) m/z 860.72 [(M+H)⁺; C₄₈H₅₈ClN₈O₅⁺ calcd for 861.42]

XL112 was synthesized according to procedure A using (*rac*)-2-benzyl-5-((tert-butoxycarbonyl)amino)pentanoic acid in step 4 and 9-chloro-5,6,7,8-tetrahydroacridine-3-carboxylic acid in step 5.

***N*-(4-benzyl-5-(4-hydroxy-4-((7-(3-(4-methylpiperazin-1-yl)propanamido)-4-oxoquinazolin-3(4H)-yl)methyl)piperidin-1-yl)-5-oxopentyl)-9-chloro-5,6,7,8-tetrahydroacridine-3-carboxamide** ¹H NMR (500 MHz, DMSO) δ 10.50 (d, *J* = 5.5 Hz, 1H), 8.82 – 8.70 (m, 1H), 8.46 (d, *J* = 13.8 Hz, 1H), 8.22 – 7.93 (m, 5H), 7.60 (dd, *J* = 16.4, 9.4 Hz, 1H), 7.29 – 7.03 (m, 5H), 4.82 (s, 1H), 4.06 (dd, *J* = 62.6, 12.8 Hz, 1H), 3.94 – 3.73 (m, 1H), 3.62 (m, 2H), 3.28 (m, 4H, overlapped with H₂O), 3.13 (t, *J* = 11.0 Hz, 2H), 3.05 (m, 2H), 2.96 (d, *J* = 11.0 Hz, 2H), 2.85 (m, 1H), 2.79 – 2.71 (m, 2H), 2.70 – 2.60 (m, 4H), 2.53 (m, 3H), 2.14 (s, 4H), 1.88 (d, *J* = 3.1 Hz, 4H), 1.71 – 1.34 (m, 5H), 1.33 – 1.03 (m, 4H), 0.39 (m, 1H). ¹³C NMR (126 MHz, DMSO) δ 172.74/172.69(conformer), 171.51, 165.80/165.76(conformer), 160.89/160.84(conformer), 160.58/160.52(conformer), 149.82/149.78(conformer), 149.46, 146.10/146.07(conformer), 144.75, 140.47,

140.32/140.25(conformer), 135.68, 130.58/130.53(conformer), 129.43/129.29(conformer), 128.63/128.51(conformer), 127.93/127.87(conformer), 127.77/127.70(conformer), 126.50/126.44(conformer), 126.29, 125.87/125.83(conformer), 123.90, 118.75, 117.07/116.95(conformer), 115.17, 69.67/69.59(conformer), 55.22, 54.04/53.84(conformer), 53.99, 52.82, 46.19, 41.97/41.74,(conformer) 41.41/41.19(conformer), 39.15, 37.51/37.46(conformer), 35.73, 35.13, 34.97, 34.78, 34.47, 34.06, 30.97, 30.16, 29.48, 27.56, 27.26/27.23(conformer), 22.36. LCMS (ESI) m/z 860.72 [(M+H)⁺; C₄₈H₅₈ClN₈O₅⁺ calcd for 861.42]

XL058 was synthesized according to procedure A using (*rac*)-2-benzyl-5-((tert-butoxycarbonyl)amino)pentanoic acid in step 4 and 5,6,7,8-tetrahydroacridine-3-carboxylic acid in step 5.

***N*-(4-benzyl-5-(4-hydroxy-4-((7-(3-(4-methylpiperazin-1-yl)propanamido)-4-oxoquinazolin-3(4H)-yl)methyl)piperidin-1-yl)-5-oxopentyl)-5,6,7,8-tetrahydroacridine-3-carboxamide** ¹H NMR (500 MHz, DMSO) δ 10.50 (d, J = 4.4 Hz, 1H, conformer), 8.74 – 8.60 (m, 1H), 8.40 (d, J = 14.3 Hz, 1H, conformer), 8.11 (m, 2H), 8.07 – 7.96 (m, 2H), 7.92 – 7.81 (m, 2H), 7.69 – 7.52 (m, 1H), 7.16 (m, 5H), 4.82 (s, 1H, conformer), 4.06 (dd, J = 61.0, 12.9 Hz, 1H, conformer), 3.88 (q, J = 13.9 Hz, 1H), 3.71 (dd, J = 88.4, 13.6 Hz, 3H), 3.27 (m, 2H), 3.13 (m, 2H), 3.03 (dd, J = 7.9, 4.8 Hz, 2H), 2.96 (m, 2H), 2.83 (m, 1H), 2.80 – 2.69 (m, 2H), 2.69 – 2.59 (m, 4H), 2.59 – 2.52 (m, 3H), 2.16 (d, J = 18.6 Hz, 3H), 1.98 – 1.87 (m, 2H), 1.87 – 1.77 (m, 2H), 1.60 (d, J = 29.3 Hz, 1H), 1.43 (m, 4H), 1.35 – 1.00 (m, 4H), 0.40 (t, J = 10.7 Hz, 1H). ¹³C NMR (126 MHz, DMSO) δ 172.76/172.70(conformer), 171.52, 166.30, 160.61/160.52(conformer), 160.32/160.30(conformer), 149.84/149.79(conformer), 149.46, 145.88/145.85(conformer), 144.75, 140.49, 140.25, 134.81, 134.75, 132.73/132.70(conformer), 129.44/129.30(conformer), 128.62/128.51(conformer), 127.78/127.75(conformer), 127.62, 127.42/127.37(conformer), 126.49/126.44(conformer), 124.42/124.38(conformer), 118.76, 117.07/116.97(conformer), 115.17, 69.67/69.59(conformer), 55.23, 54.04/53.84(conformer), 54.00, 52.84, 46.20, 41.97/41.74(conformer), 41.40/41.17(conformer), 39.18, 37.46, 35.72, 35.13, 34.95, 34.79, 34.47, 33.46, 30.97, 30.17, 29.03, 27.31, 23.05, 22.75. LCMS (ESI) m/z 827.61 [(M+H)⁺ C₄₈H₅₉N₈O₅⁺ calcd for 827.46]

XL041 was synthesized according to procedure A using 5-((tert-butoxycarbonyl)amino)pentanoic acid in step 4 and 9-chloro-5,6,7,8-tetrahydroacridine-3-carboxylic acid in step 5.

***9*-chloro-*N*-(5-(4-hydroxy-4-((7-(3-(4-methylpiperazin-1-yl)propanamido)-4-oxoquinazolin-3(4H)-yl)methyl)piperidin-1-yl)-5-oxopentyl)-5,6,7,8-tetrahydroacridine-3-carboxamide** ¹H NMR (500 MHz, DMSO) δ 10.60 (s, 1H), 8.79 (t, J = 5.0 Hz, 1H), 8.46 (s, 1H), 8.22 (s, 1H), 8.15 (d, J = 8.7 Hz, 1H), 8.06 (dd, J = 14.6, 8.7 Hz, 3H), 7.64 (d, J = 8.8 Hz, 1H), 4.11 – 3.90 (m, 4H), 3.65 (d, J = 13.0 Hz, 1H), 3.38 – 3.20 (m, 6H), 3.07 (m, 5H), 2.96 (m, 4H), 2.77 (m, 6H), 2.36 (m, 2H), 1.88 (s, 3H), 1.55 (d, J = 26.5 Hz, 6H), 1.48 – 1.31 (m, 4H). ¹³C NMR (126 MHz, DMSO) δ 170.76, 170.28, 165.81, 160.83, 160.63, 149.94, 149.38, 145.93, 144.60, 140.44,

135.78, 130.56, 127.77, 127.74, 126.26, 125.85, 123.90, 118.84, 117.12, 115.34, 69.80, 53.86, 52.38, 51.73, 49.40, 42.61, 41.48, 39.60, 37.41, 35.58, 34.82, 33.98, 33.18, 32.45, 29.17, 27.54, 22.89, 22.33. LCMS (ESI) m/z 771.47 [(M+H)⁺; C₄₁H₅₂ClN₈O₅⁺ calcd for 771.37]

Synthesis of XL024 (see Figure S16):

Step 1 (Synthesis of **S5**): 7-nitroquinazolin-4(3*H*)-one (0.77g, 4.0mmol) and tert-butyl 4-(bromomethyl)piperidine-1-carboxylate (1.11g, 4.0mmol) were added into 10mL DMF. Cesium carbonate (3.90g, 12.0mmol) was added in one portion. The mixture was heated at 80°C overnight. The mixture was diluted with EtOAc, then washed with sat. NaCl. Combined organic layer was concentrated under reduced pressure. The crude product was purified by flash chromatography (EtOAc in hexanes: 40%-100%) to afford 1.14g **S5** (73%). ¹H NMR (500 MHz, CDCl₃) δ 8.54 (d, *J* = 2.0 Hz, 1H), 8.45 (d, *J* = 8.8 Hz, 1H), 8.26 (dd, *J* = 8.8, 2.1 Hz, 1H), 8.04 (s, 1H), 4.14 (br, 2H), 3.89 (br, 2H), 2.65 (m, 2H), 2.05 (m, 1H), 1.65 (d, *J* = 12.4 Hz, 2H), 1.43 (s, 9H), 1.24 (qd, *J* = 12.6, 4.3 Hz, 2H). LCMS (ESI) m/z 332.87 (M+H-*t*-Butyl) [(M+H)⁺ C₁₉H₂₅N₄O₅⁺ calcd for 389.18]

Step 2 (Synthesis of **S6**): Compound **S5** (1.14g, 2.9mmol) was suspended in 10mL solvent (EtOH/AcOH=1:1). 4 eq. of Fe powder was added in portions. The mixture was stirred for 2 hour at 50°C. Then the reaction was cooled down to room temperature, and filtered through a pad of Celite. The filtrate was concentrated under reduced pressure to afford the crude product, which was then purified by flash chromatography (MeOH in EtOAc: 0% to 70%) to afford 1.07g product **S6** (quant.) ¹H NMR (500 MHz, CDCl₃) δ 8.09 – 8.03 (dd, *J* = 7.5, 1.5 Hz, 1H), 7.85 (s, 1H), 6.80 – 6.74 (m, 2H), 4.23 (br, 2H), 4.10 (br, 2H), 3.78 (br, 2H), 2.63 (m, 2H), 2.06 – 1.97 (m, 1H), 1.64 (m, 2H), 1.45 – 1.39 (s, 9H), 1.20 (qd, *J* = 12.5, 4.0 Hz, 2H). LCMS (ESI) m/z 302.97 (M+H-*t*-Butyl) [(M+H)⁺ C₁₉H₂₇N₄O₃⁺ calcd for 359.21]

Step 3 (Synthesis of **S7**): Compound **S6** (0.6g, 1.68mmol) was dissolved in 5mL anhydrous dichloromethane under N₂ at 0°C. 3.0 eq. of Et₃N was added. Then 3-bromopropionyl chloride (0.35g, 2.1mmol) was added dropwise. The mixture was stirred at 0°C for 3 hours, then quenched with MeOH, and concentrated under reduced pressure. The solid residue was directly used for the following step without further purification. The crude product from last step was dissolved in 10mL MeOH, then 3.0eq of Et₃N was added. Into the stirred mixture was added 1-methylpiperazine (0.26g, 2.52mmol) dropwise. After the addition completed, the mixture was stirred at 50°C overnight. Then the reaction mixture was cooled down to room temperature, then directly subjected to flash chromatography purification (MeOH in EtOAc: 0% to 70%) to afford 0.72g product **S7** (83% in two steps) ¹H NMR (500 MHz, CDCl₃) δ 11.42 (s, 1H), 8.22 (d, *J* = 8.7 Hz, 1H), 7.91 (s, 1H), 7.84 (d, *J* = 2.0 Hz, 1H), 7.72 (dd, *J* = 8.7, 2.0 Hz, 1H), 4.11 (s, 2H), 3.83 (s, 2H), 2.74 (m, 4H), 2.64 (m, 4H), 2.59 – 2.46 (m, 4H), 2.36 (s, 3H),

2.13 – 1.98 (m, 1H), 1.65 (m, 4H), 1.43 (s, 9H), 1.21 (qd, $J = 12.4, 4.2$ Hz, 2H). LCMS (ESI) m/z 512.98 [(M+H)⁺ C₂₇H₄₁N₆O₄⁺ calcd for 513.32].

Step 4 (Synthesis of S8): S7 (0.17g, 0.32mmol) was dissolved in 3mL TFA. The solution was stirred for 1 hour at room temperature. Then the mixture was concentrated under reduced pressure, and left on high vacuum overnight to remove residual acid. Then the product (0.14g, 0.32mmol) was dissolved in 3mL DMF, and basified by adding 10 eq of Et₃N. Into the solution 2-benzyl-5-((tert-butoxycarbonyl)amino)pentanoic acid (0.14g, 0.48mmol) and HATU (0.24g, 0.64mmol) sequentially. The resultant solution was stirred overnight. Then the mixture was directly subjected to flash chromatography purification (MeOH in EtOAc: 0% to 70%) to afford 0.23g S8 (quantitative) ¹H NMR (500 MHz, DMSO) δ 10.52 (s, 1H), 8.27 and 8.13 (s, 1H, conformer), 8.07 (d, $J = 8.5$ Hz, 1H, conformer), 8.04 – 7.99 (d, $J = 7.5$ Hz, 1H, conformer), 7.67 – 7.58 (m, 1H), 7.25 (m, 2H), 7.19 – 7.08 (m, 3H), 6.83 – 6.71 (m, 1H), 4.37 (d, $J = 12.5$ Hz, 1H, conformer), 3.82 (m, 2H), 3.64 – 3.57 (dd, $J = 13.5, 7.5$ Hz, 1H, conformer), 3.05 (m, 1H), 2.96 – 2.80 (m, 2H), 2.80 – 2.68 (m, 2H), 2.62 (m, 3H), 2.54 (m, 2H), 2.37 (m, 8H), 2.16 (s, 3H), 2.00 – 1.81 (m, 1H), 1.52 (m, 1H), 1.37 (m, 11H), 1.31 – 1.19 (m, 2H), 1.13 – 0.98 (m, 1H), 0.71 and -0.03 (dd, $J = 21.2, 11.8$ Hz, 1H, conformer). LCMS (ESI) m/z 701.90 [(M+H)⁺ C₃₉H₅₆N₇O₅⁺ calcd for 702.43]

Step 5 (Synthesis of XL024): S8 (0.13g, 0.18mmol) was dissolved in 4M HCl in 1,4-dioxane, and stirred for 1h at room temperature. Then the mixture was concentrated under reduced pressure, and left on high vacuum overnight to remove residual solvent. Then the product (0.11g, 0.18mmol) was dissolved in 5mL anhydrous DCM with 10 eq. of Et₃N. Into the solution was added 9-chloro-5,6,7,8-tetrahydroacridine-3-carboxylic acid (0.05g, 0.2mmol), and T3P (50% in EtOAc) (0.17g, 0.54mmol). The solution was stirred at room temperature under nitrogen overnight. Then the mixture was concentrated under reduced pressure, and purified sequentially by flash chromatography (MeOH in EtOAc: 0% to 70%) and HPLC (MeOH/H₂O with 4‰ TFA) to afford 50mg XL024 (33%).

(4-benzyl-5-(4-((7-(3-(4-methylpiperazin-1-yl)propanamido)-4-oxoquinazolin-3(4H)-yl)methyl)piperidin-1-yl)-5-oxopentyl)-9-chloro-5,6,7,8-tetrahydroacridine-3-carboxamide ¹H NMR (500 MHz, DMSO) δ 10.52 (d, $J = 4.2$ Hz, 1H, conformer), 8.78 (dt, $J = 18.3, 5.4$ Hz, 1H, conformer), 8.48 (d, $J = 18.1$ Hz, 1H, conformer), 8.23 – 8.11 (m, 2H), 8.11 – 7.99 (m, 3H), 7.62 (m, 1H), 7.19 (m, 5H), 4.39 (dd, $J = 39.4, 12.8$ Hz, 1H, conformer), 3.78 (m, 2H), 3.61 (ddd, $J = 43.3, 13.4, 7.0$ Hz, 1H), 3.29 (m, 2H), 3.13 (s, 1H), 3.06 (m, 2H), 2.99 (m, 2H), 2.88 – 2.71 (m, 2H), 2.71 – 2.59 (m, 4H), 2.58 – 2.52 (m, 3H), 2.39 (m, 4H), 2.15 (s, 3H), 2.00 – 1.83 (m, 5H), 1.74 – 1.35 (m, 6H), 1.23 (m, 1H), 1.05 (td, $J = 23.0, 11.6$ Hz, 1H), 0.72 (dt, $J = 12.3, 8.8$ Hz, 1H). ¹³C NMR (126 MHz, DMSO) δ 172.78/172.70(conformer), 171.53, 165.81/165.75(conformer), 160.91, 160.17, 149.45, 148.92,

146.06, 144.77, 140.50, 140.33/140.28(conformer), 135.68, 130.59, 129.58/129.32(conformer), 128.69/128.51(conformer), 127.89/127.87(conformer), 127.54/127.49(conformer), 126.51/126.44(conformer), 126.32/126.29(conformer), 125.87/125.84(conformer), 123.96/123.91(conformer), 118.94, 117.03/116.98(conformer), 115.25, 55.23, 54.00, 52.84, 50.97/50.80(conformer), 46.20, 45.31, 44.72, 42.09/41.83(conformer), 41.54, 41.16, 39.01, 35.56/35.39(conformer), 34.79, 34.07, 30.92, 30.30, 29.98, 29.61, 29.35, 27.56, 27.28/27.18(conformer), 22.36. LCMS (ESI) m/z 844.82 [(M+H)⁺ C₄₈H₅₈ClN₈O₄⁺ calcd for 845.43]