First-in-class metallo-PROTAC as an effective degrader of select Pt-binding proteins

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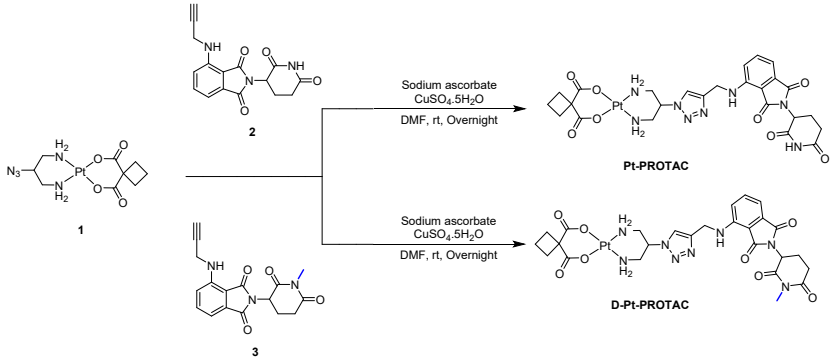
Supplementary Information

Materials & Instrumentation

All chemicals and solvents were purchased from Sigma Aldrich (*Sigma Aldrich Ireland Ltd, Co. Wicklow, Ireland*) unless stated otherwise, and were used without further purification. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker Avance 400 MHz NMR spectrometer. All spectra were analysed using MestReNova software and the residual undeuterated solvent signals were used as internal references. High resolution electrospray ionization (ESI) mass spectrometry was carried out in positive mode on a Bruker Compact™ mass spectrometer and analysed using mMass software. High performance liquid chromatography was performed with a Shimazdu SIL-20AHT HPLC instrument equipped with a Shimazdu SPD-20AV prominence UV/Vis detector. Analytical separation employed a Gemini column; size: 250 x 4.6 mm, I.D. particle size: 5 µm, detection method: UV-Vis, wavelength for detection: 400 nm. Eluent: 0-10 min (10% ACN to 50% ACN), 10-15 min (50% ACN), 15-20 min (50% ACN to 10% ACN), 20-25 min (10% ACN) with 1.0 ml/min flow rate, oven temperature of 25 °C and injection volume of 25 µL. Retention time; 16.578 min (Pt-PROTAC) and 18.472 min (D-Pt-PROTAC).

Syntheses

[Pt(DAP-N_3)(CBDCA$_{-2H}$)] **1** and pomalidomide analogues **2** and **3** were synthesised as previously reported. [1, 2]



Synthesis of Pt-PROTAC

1 (160 mg) and **2** (178 mg, 1.5 equiv.) were dissolved in 5 mL DMF. To this was added a mixture of copper sulphate pentahydrate (36.1 mg, 0.38 equiv.) and sodium ascorbate (18.1 mg, 0.24 equiv.) in 2 mL DMF. The resulting mixture was stirred in the dark at room temperature overnight and then C18 silica was added to the mixture. The solvent was removed from the mixture under reduced pressure and the resulting powder dry-loaded for C18 silica gel

chromatography (90:10 H_2O:Acetonitrile to 50: 50 H_2O: Acetonitrile). Product fractions were identified by silica gel TLC (90:10 DCM:MeOH) and then combined. The combined fractions were washed with ethyl acetate (3 x 30 mL) to remove any remaining organic impurities and the aqueous layer dried by lyophilisation. (80 mg, 27.5% yield, bright yellow powder). ^{1}H NMR (400 MHz, DMSO-d6) δ 11.11 (s, 1H), 8.15 (s, 1H), 7.57 (t, 1H, *J* = 7.8 Hz), 7.14 (d, 1H, *J* = 8.6 Hz), 7.07 (m, 2H), 5.81 (d, 2H, *J* = 10.8 Hz), 5.58 – 5.33 (m, 2H), 5.06 (dd, 1H, *J* = 12.8, 5.3 Hz), 4.60 (m, 3H), 2.98 – 2.81 (m, 3H), 2.75 – 2.58 (m, 5H), 2.58 – 2.51 (m, 3H), 2.07 – 1.97 (m, 1H), 1.72 – 1.60 (m, 2H). ^{13}C NMR (101 MHz, DMSO-d6) δ 177.4, 172.8, 170.1, 168.8, 167.3, 145.8, 145.0, 136.2, 132.1, 121.8, 117.6, 111.0, 109.7, 58.0, 55.6, 48.6, 47.4, 37.7, 31.0, 30.5, 30.4, 22.2, 15.0. HRMS (ESI$^+$) (H_2O/MeOH) [M + Na]$^+$: $C_{25}H_{28}N_8NaO_8Pt$, calc.: 786.1576, found: 786.1574. Purity (HPLC): 95.3%.

Synthesis of D-Pt-PROTAC

1 (80 mg) and **3** (93 mg, 1.5 equiv.) were dissolved in 5 mL DMF. To this was added a mixture of copper sulphate pentahydrate (18 mg, 0.38 equiv.) and sodium ascorbate (9 mg, 0.24 equiv.) in 2 mL DMF. The resulting mixture was stirred in the dark at room temperature overnight and then C18 silica was added to the mixture. The solvent was removed from the mixture under reduced pressure and the resulting powder dry-loaded for C18 silica gel chromatography (90:10 H_2O:Acetonitrile to 50: 50 H_2O: Acetonitrile). Product fractions were identified by silica gel TLC (90:10 DCM:MeOH) and then combined. The combined fractions were washed with ethyl acetate (3 x 30 mL) to remove any remaining organic impurities and the aqueous layer dried by lyophilisation. (36 mg, 24.3% yield, bright yellow powder). ^{1}H NMR (400 MHz, DMSO-d6) δ 8.15 (s, 1H), 7.58 (t, 1H, *J* = 7.8 Hz), 7.15 (d, 1H, *J* = 8.6 Hz), 7.08 (m, 2H), 5.82 (d, 2H, *J* = 10.9 Hz), 5.59 – 5.37 (m, 2H), 5.13 (dd, 1H, *J* = 13.0, 5.3 Hz), 4.59 (m, 3H), 3.01 (s, 3H), 3.00 – 2.86 (m, 3H), 2.76 (m, 1H), 2.67 (m, 4H), 2.56 (m, 3H), 2.09 – 2.00 (m, 1H), 1.71 – 1.60 (m, 2H). ^{13}C NMR (101 MHz, DMSO-d6) δ 177.4, 171.8, 169.8, 168.8, 167.3, 145.8, 145.0, 136.2, 132.1, 121.8, 117.6, 111.0, 109.7, 58.0, 55.6, 49.1, 47.4, 37.7, 31.1, 30.6, 30.3, 26.6, 21.4, 15.0. HRMS (ESI$^+$) (H_2O/MeOH) [M + Na]$^+$: $C_{26}H_{30}N_8NaO_8Pt$, calc.: 800.1732, found: 800.1976. Purity (HPLC): 97.8%.

Biological Evaluations

Cell Culture

JJN3 and MM1.S cell lines were cultured in RPMI media (Gibco), supplemented with 10% foetal calf serum (FCS), L-glutamine (2 mM) and penicillin-streptomycin (100U/ml, 100 μg/ml). All cell lines were cultured at 37°C in a humidified atmosphere with 5% CO_2. For apoptosis assays, cells were plated at a density of 5x10^4 cells/well in 24-well or 0.5-1.0 x 10 24-well plates and cultured for a further 24h before treatments. For western immunoblotting assays, cells were plated at a density of 4 x 10^5 cells/well in 6-well plates and cultured for a further 24h before treatments.

Apoptosis Assays

Cells (5 x 10^4 cells /well in 24-well plates) were treated with DMSO, carboplatin, Pt-PROTAC, deactivated Pt-PROTAC (D-Pt-PROTAC) or bortezomib at the indicated concentrations, as indicated. After 24 h apoptosis was observed based on cell morphology (cell rounding, nuclear condensation and presence of apoptotic bodies). Cell death in was then quantified using Annexin V-FITC / propidium iodide (PI) staining analysed by flow cytometry. Irrespective of the cell line being evaluated, the assay is based on incubation of cells in a solution containing Ca^{2+} ions and annexin-FITC (typically at a final concentration of 1 μg/ml) at 4^0C. The annexin V-binding buffer consists of 10 mM HEPES-NaOH, pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, and 1.8 mM $CaCl_2$. After the incubation period propidium iodide (10 mg/ml) is added to facilitate the identification of cells undergoing secondary necrosis.

Western Immunoblotting

Cells were lysed to extract protein and protein concentrations determined by Bicinchoninic acid (BCA) assay and normalised to 1mg/ml and a final amount of 20 μg per well loaded in subsequent electrophoresis steps. Protein samples were prepared using SDS/PAGE loading buffer (2 % SDS, 50 mM Tris-HCl, pH 6.8, 10 % glycerol, 2.5 % β-mercaptoethanol), boiled for 7 min and electrophoresed on 12% SDS-PAGE gels. Proteins were then transferred onto 0.2 μM nitrocellulose membranes at 250 mA 90min 4^0C. Membranes were blocked for 1 h (in 5 % NFDM, 0.05 % sodium azide in Tris-buffered saline, Tween-20, TBST). The indicated proteins were then probed using specific antibodies, typically diluted 1:1000 in 5 % NFDM. Membranes were washed 3 times in TBST and then incubated with the relevant HRP-conjugated secondary antibody diluted 1:3000 as before. Membranes were again washed and proteins were visualised by incubation with Immobilon western chemiluminescent substrate followed by exposure to LAS4000 CCD imaging system. For western blotting experiments cells (4 x 10^5/well in 6 well plates) were pre-treated for 30min in the presence or absence of bortezomib (1 nM) followed by treatment with DMSO (0.1%), carboplatin, Pt-PROTAC or D-Pt-PROTAC at concentrations indicated in Figure Legends. After 16-24 hrs treatment, as indicated, cell lysates were prepared and western immunoblotting carried out, as described above.

Reagents and antibodies

Anti-thioredoxin-1 rabbit polyclonal antibody (catalog code #2285), anti-thioredoxin reductase-1 (TRXR1) rabbit polyclonal antibody (catalog code #6925) and anti-glutathione S transferase P1 (GSTP1) mouse monoclonal antibody (catalog code #3369) were obtained from Cell Signaling. Anti-actin mouse monoclonal antibody (catalog code MAB1501) was obtained from Merck Millipore. Carboplatin, Pt-PROTAC, D-Pt-PROTAC and bortezomib (BTZ) were generated in-house.

Characterisation of Pt-PROTAC & D-Pt-PROTAC:

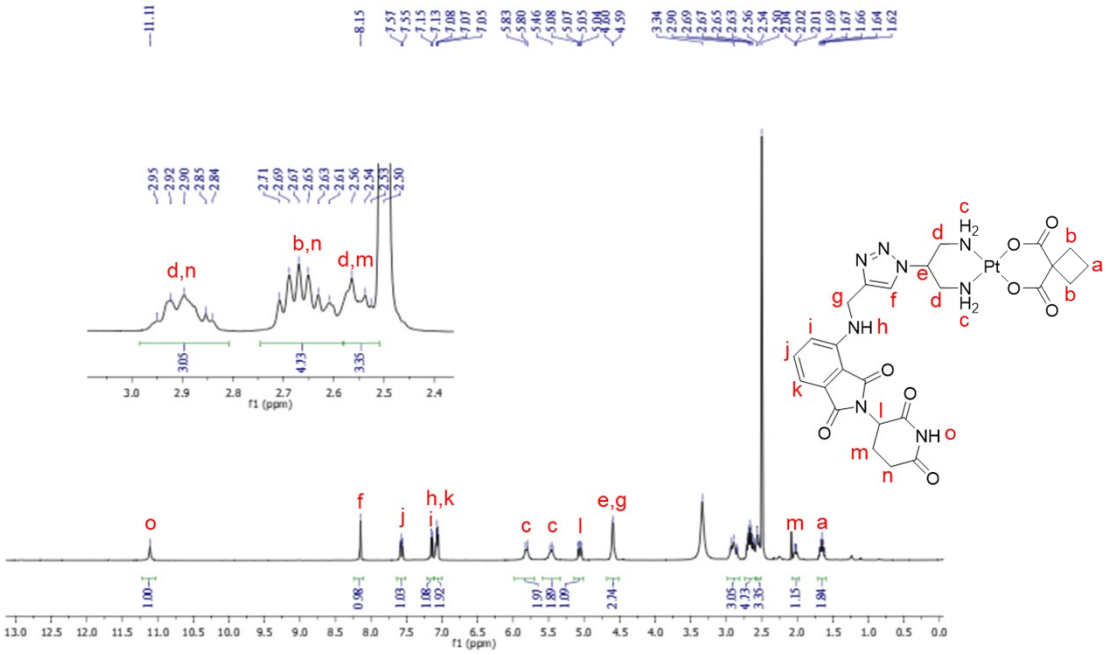


Figure S1: ^{1}H NMR of Pt-PROTAC in DMSO-*d*6.

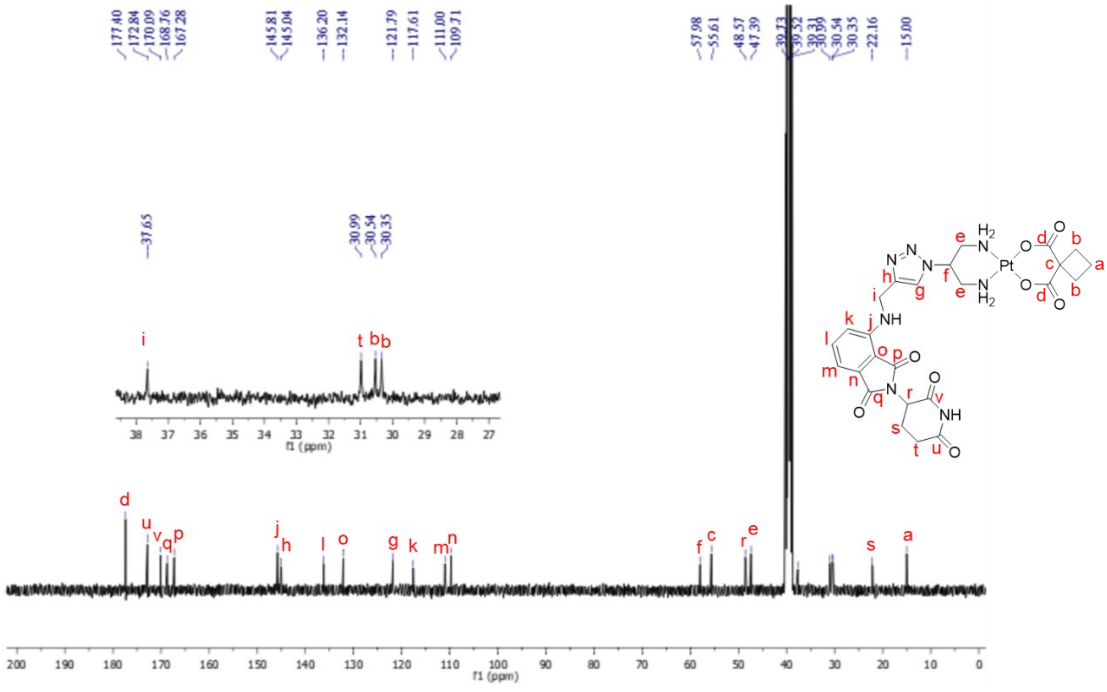


Figure S2: ^{13}C NMR of Pt-PROTAC in DMSO-*d*6.

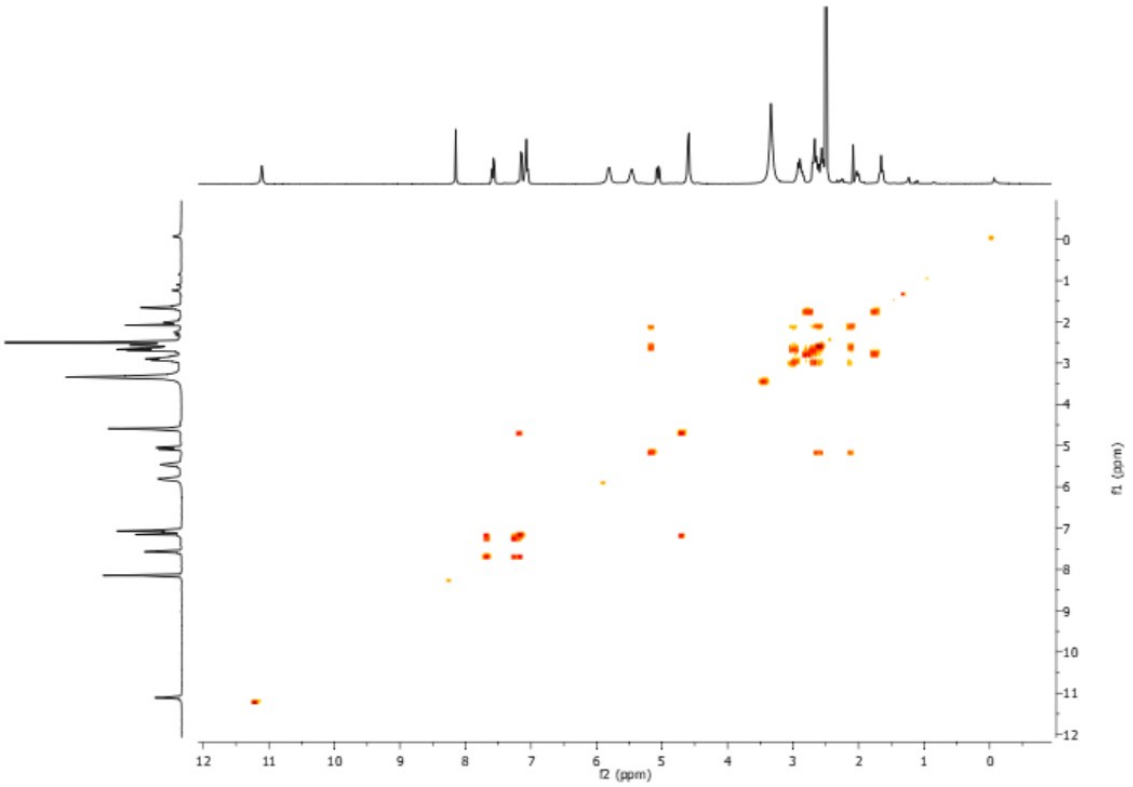


Figure S3: ^{1}H-^{1}H COSY of Pt-PROTAC in DMSO-*d*6.

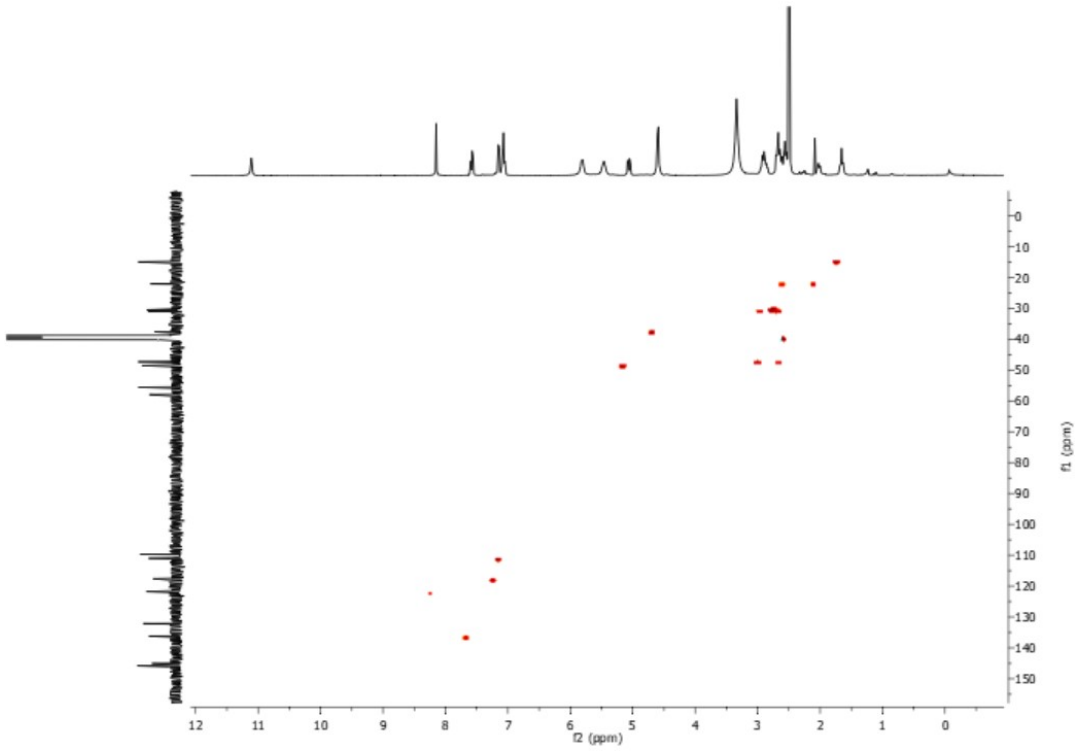


Figure S4: ^{1}H-^{13}C HSQC of Pt-PROTAC in DMSO-*d*6.

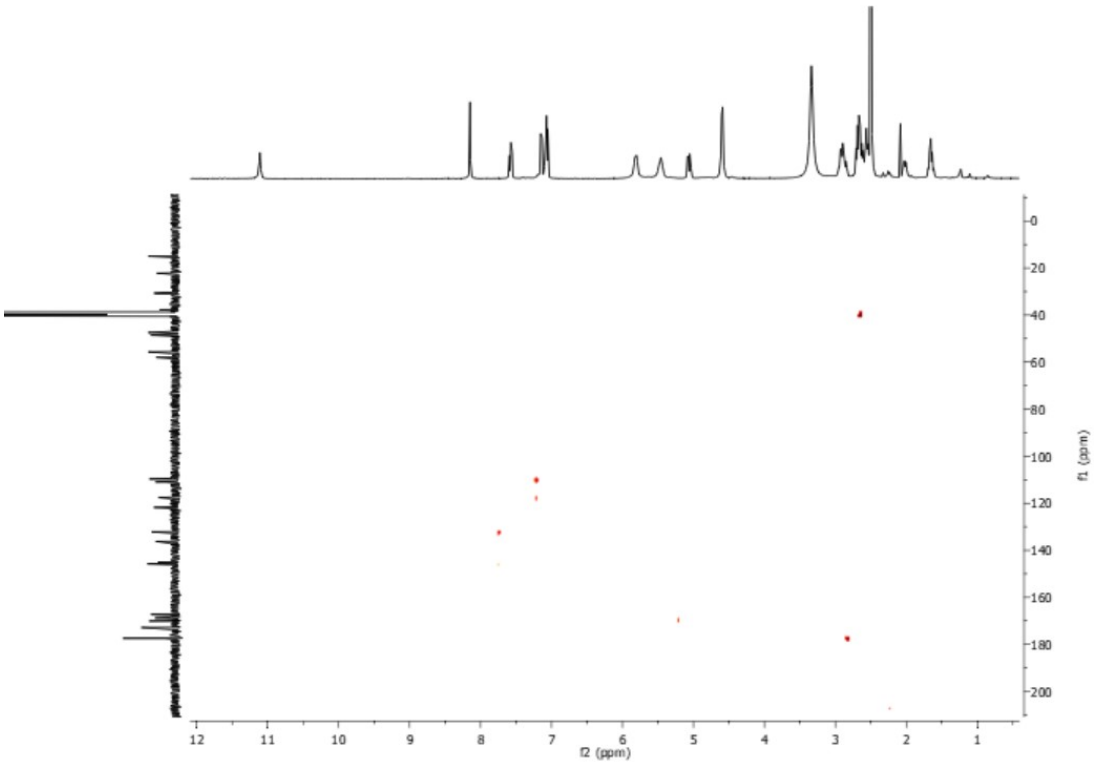


Figure S5: ^{1}H-^{13}C HMBC of Pt-PROTAC in DMSO-*d*6.

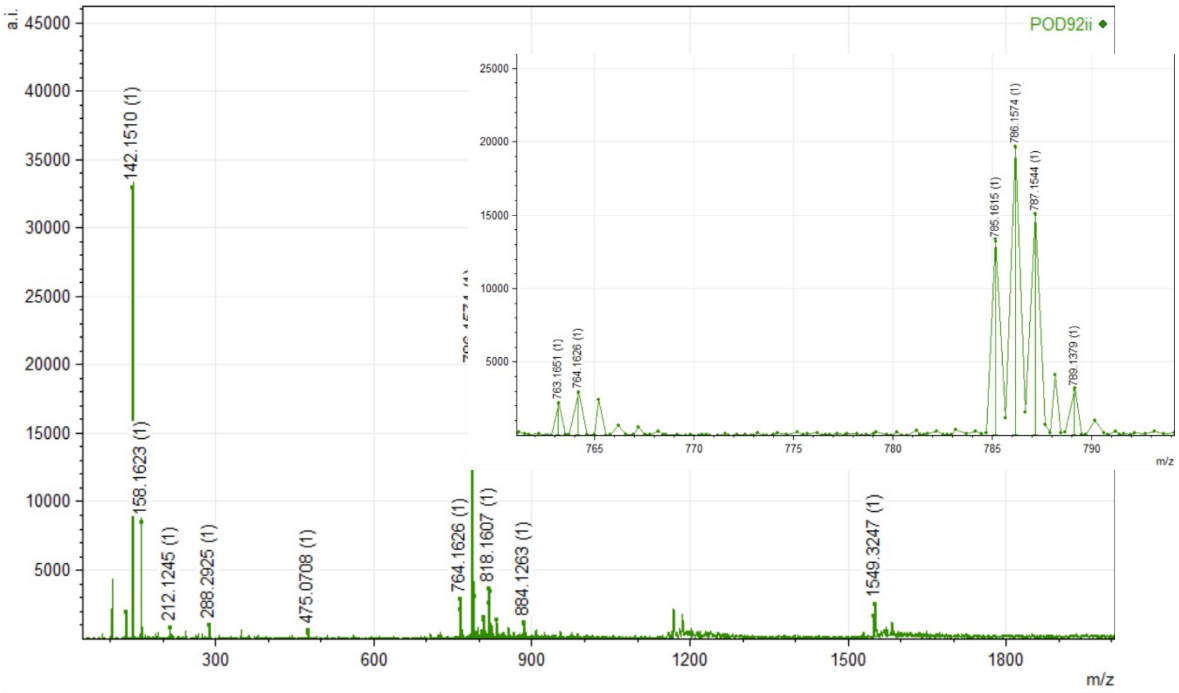


Figure S6: HRMS $(ESI)^{+}$ Spectrum of Pt-PROTAC in H_2O/MeOH

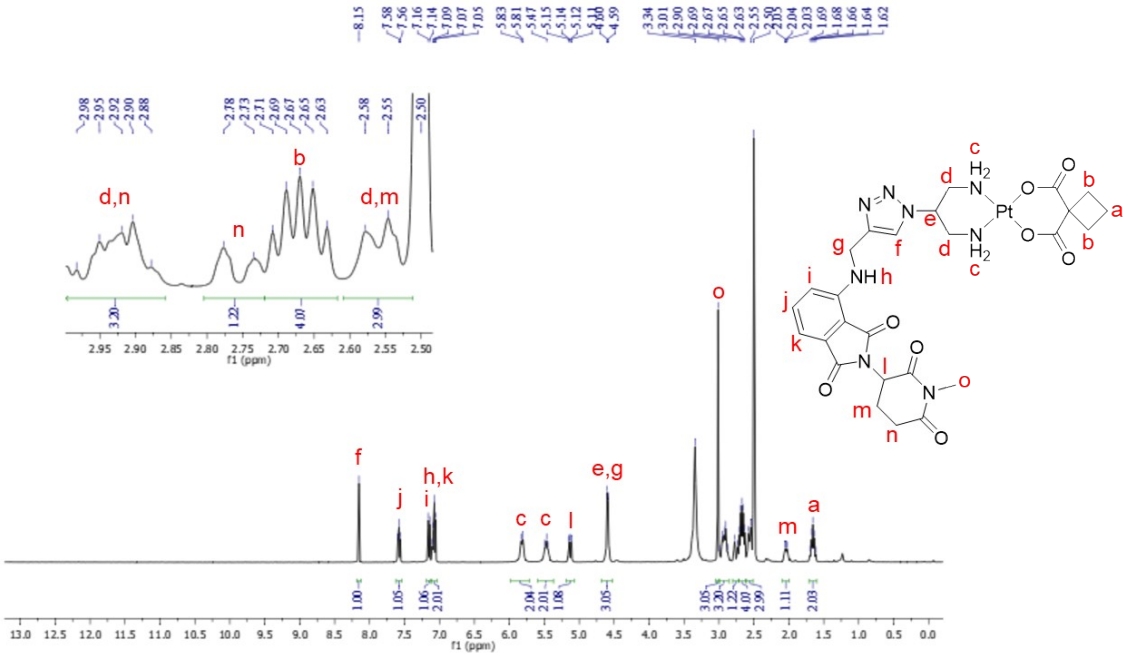


Figure S7: ^{1}H NMR of D-Pt-PROTAC in DMSO-*d*6.

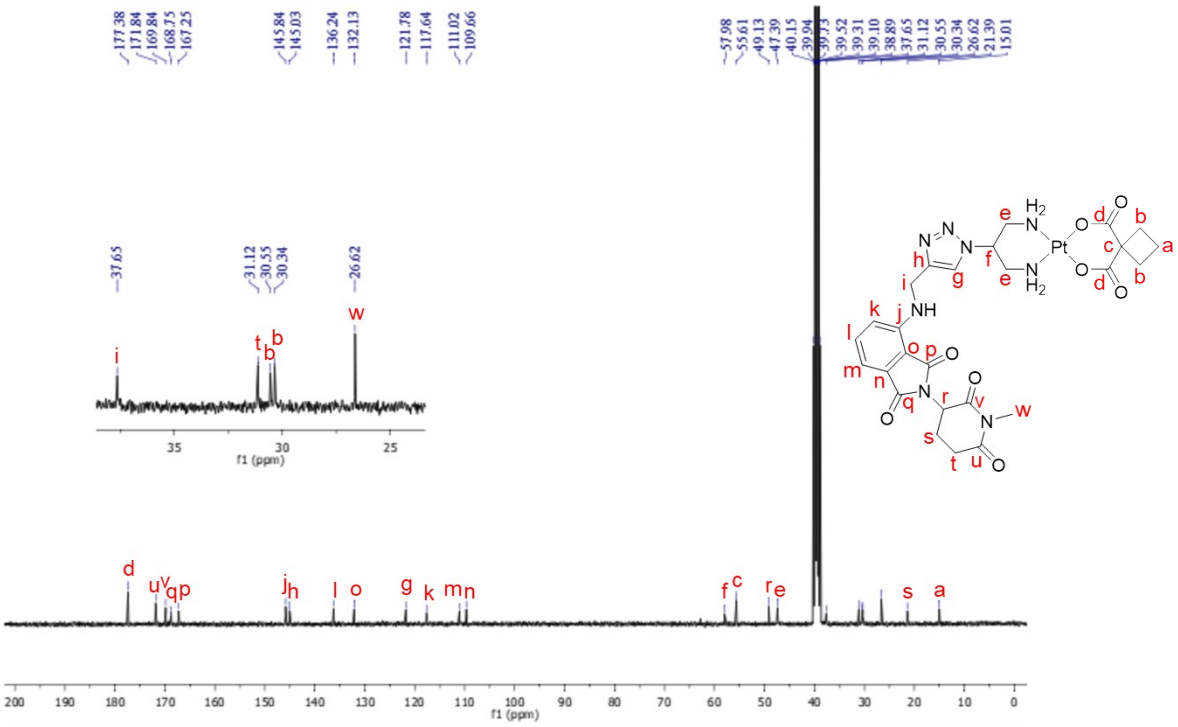


Figure S8: ^{13}C NMR of D-Pt-PROTAC in DMSO-*d*6.

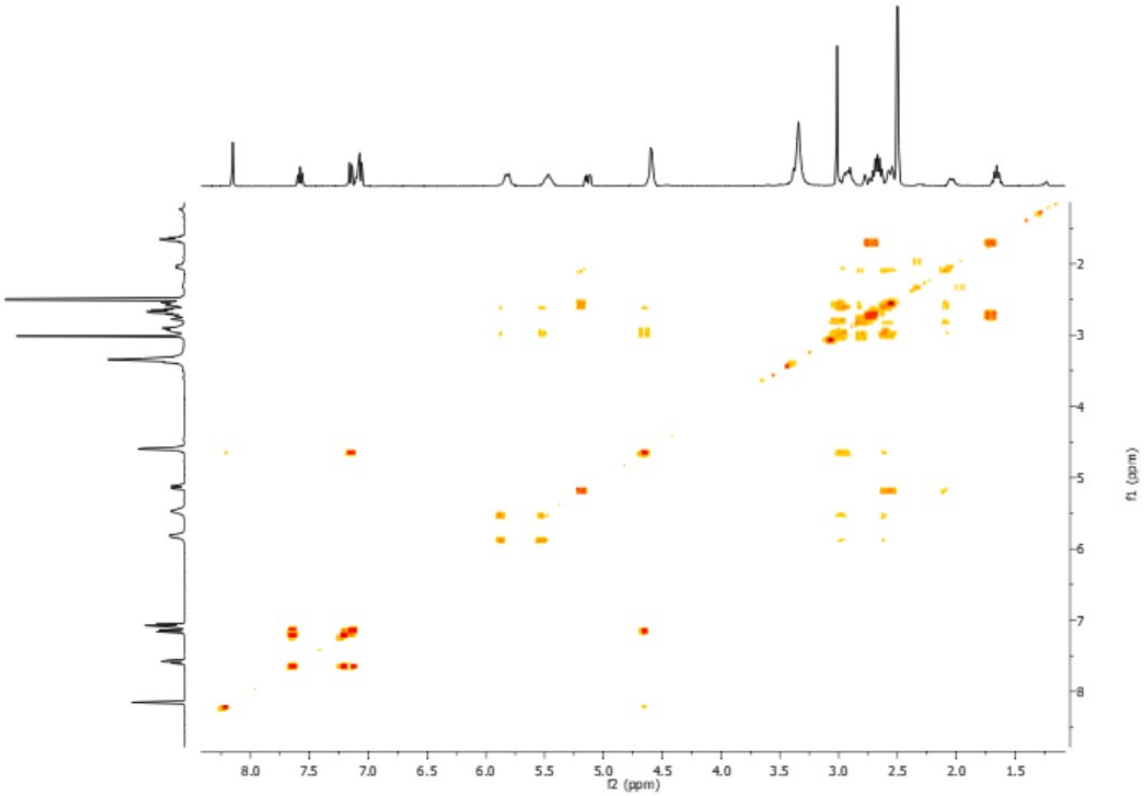


Figure S9: ^{1}H-^{1}H COSY of D-Pt-PROTAC in DMSO-*d*6.

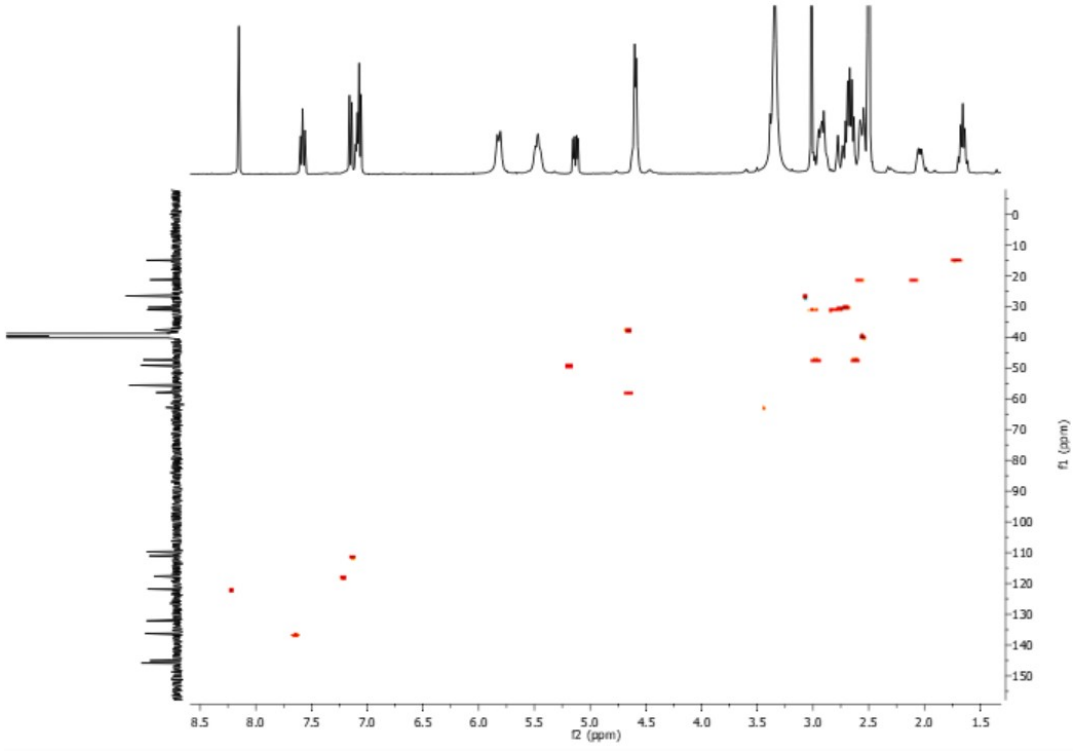


Figure S10: ^{1}H-^{13}C HSQC of D-Pt-PROTAC in DMSO-*d*6.

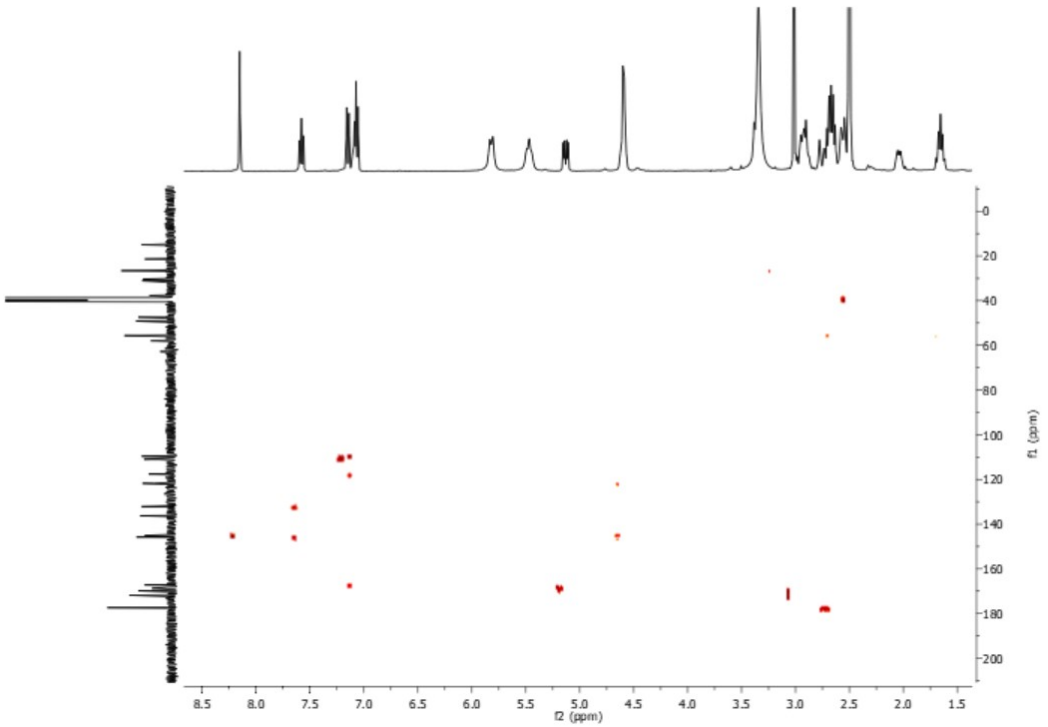


Figure S11: ^{1}H-^{13}C HMBC of D-Pt-PROTAC in DMSO-*d*6.

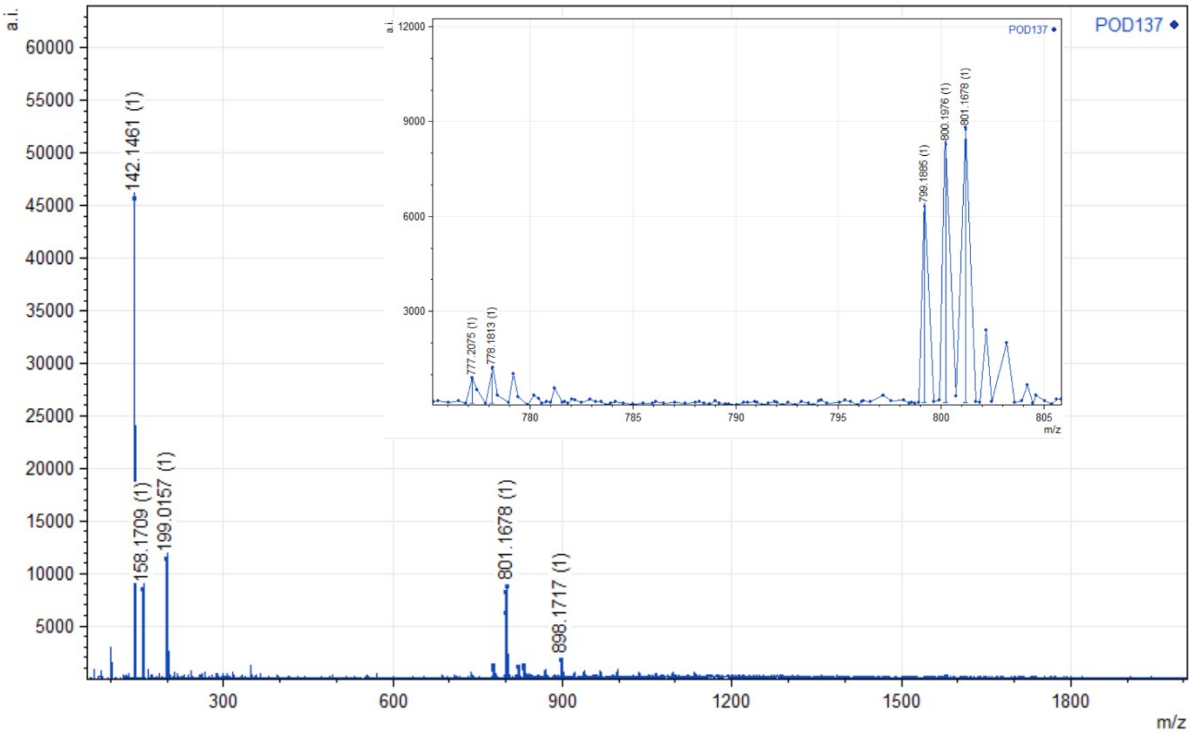


Figure S12: HRMS $(ESI)^{+}$ Spectrum of D-Pt-PROTAC in H_2O/MeOH

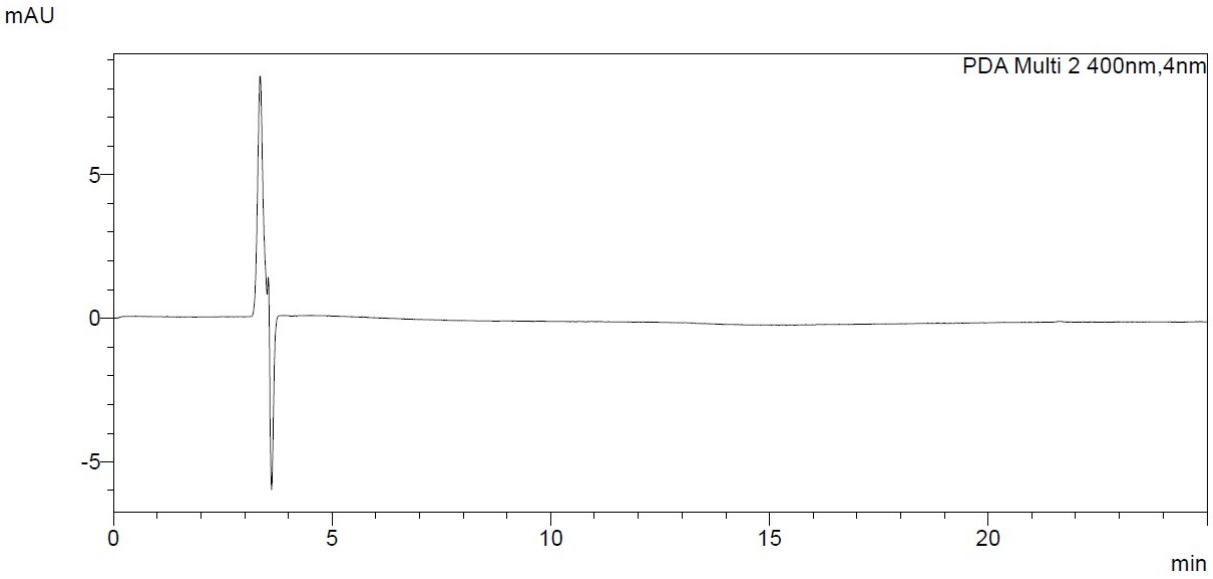
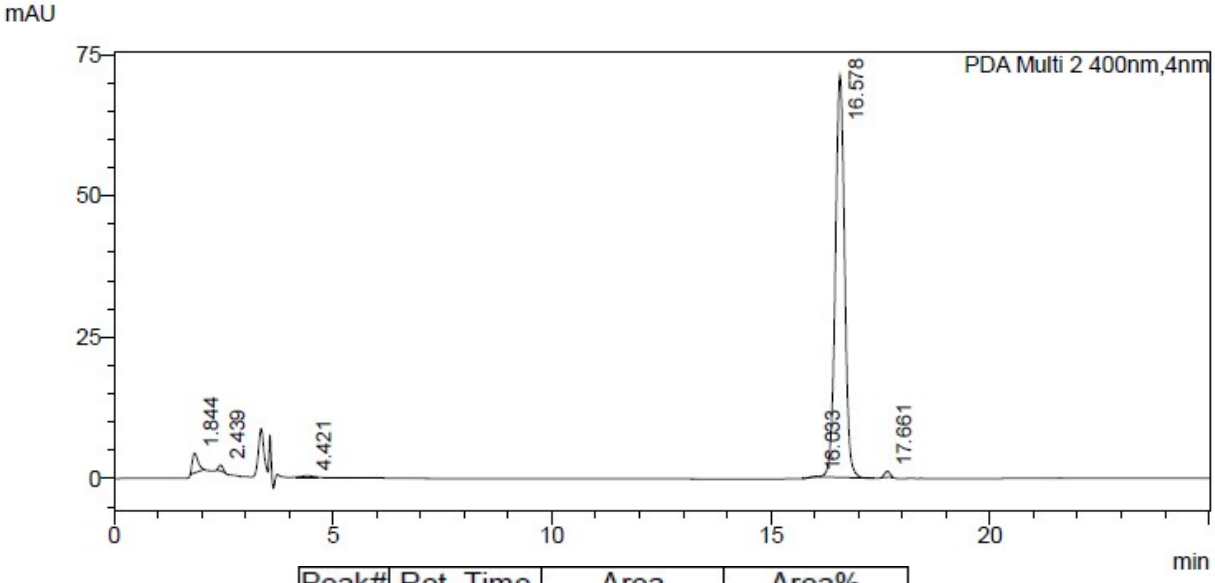
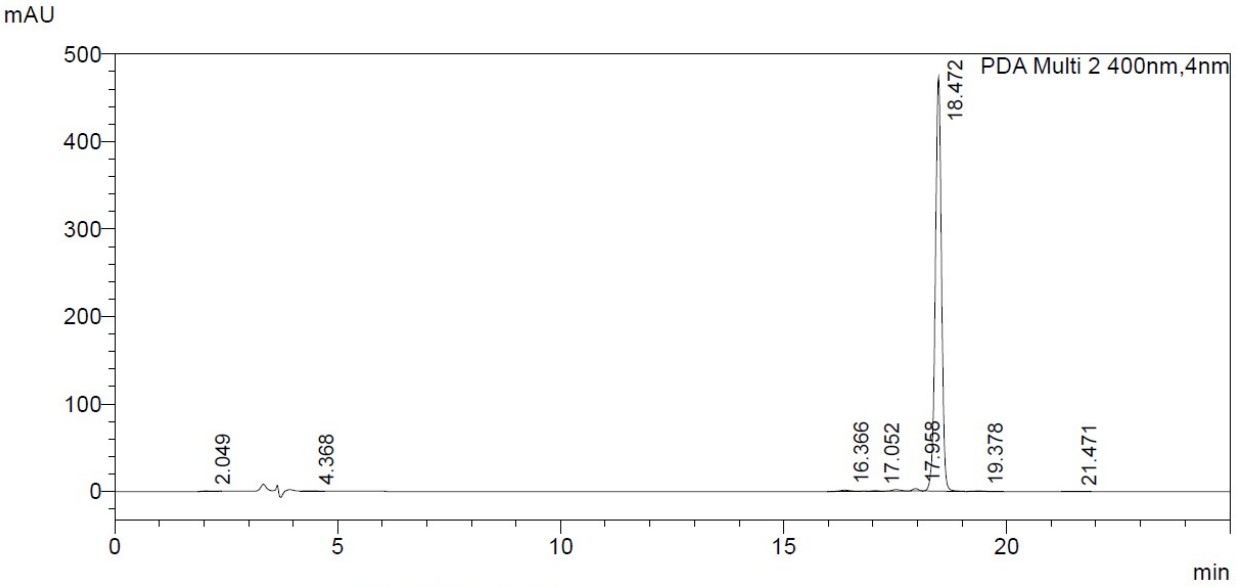


Figure S13: HPLC Chromatogram of Blank



Peak#	Ret. Time	Area	Area%
1	1.844	31383	2.861
2	2.439	6507	0.593
3	4.421	3624	0.330
4	16.033	777	0.071
5	16.578	1045594	95.328
6	17.661	8951	0.816
Total		1096835	100.000

Figure S14: HPLC Chromatogram of Pt-PROTAC. Peak at 16.578 min with 95.3% peak area corresponds to Pt-PROTAC



PDA Ch2 400nm

Peak#	Ret. Time	Area	Area%
1	2.049	4544	0.098
2	4.368	4132	0.089
3	16.366	23913	0.513
4	17.052	7705	0.165
5	17.958	50668	1.088
6	18.472	4555092	97.814
7	19.378	9159	0.197
8	21.471	1682	0.036
Total		4656892	100.000

Figure S15: HPLC Chromatogram of D-Pt-Control. . Peak at 18.472 min with 97.8% peak area corresponds to D-Pt-PROTAC

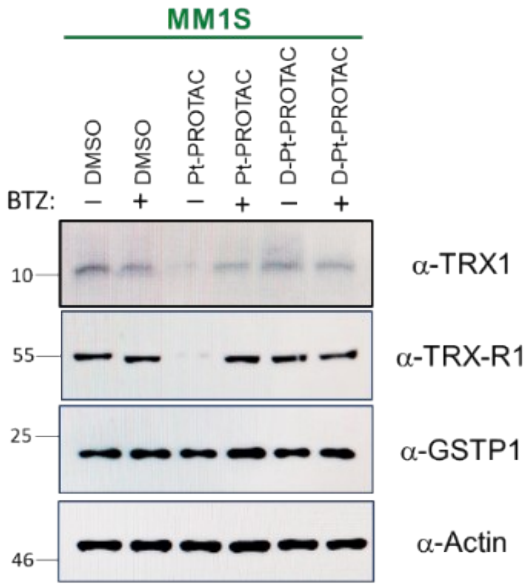
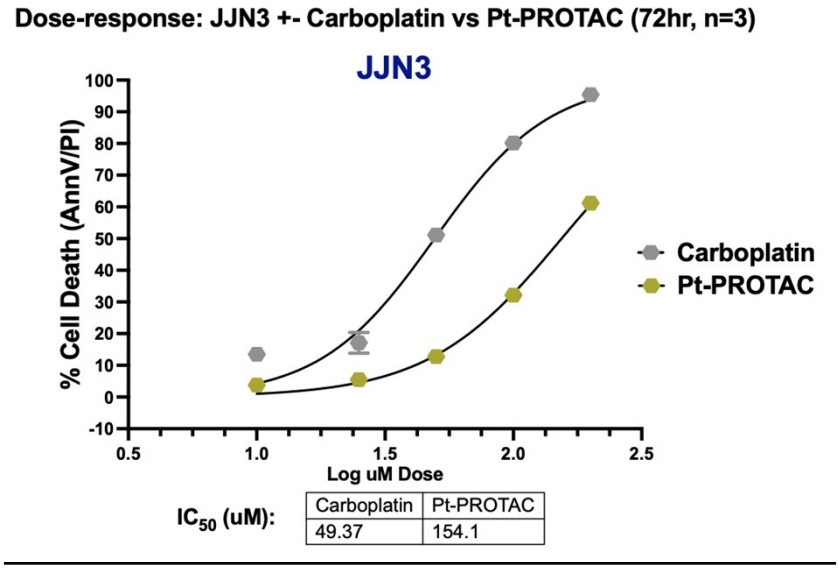


Figure S16. Western blot analysis of whole cell lysates of MM1.S cells after treatment with Pt-PROTAC, D-Pt-PROTAC and vehicle control at 100 µM for 24 h with (+) or without (-) the presence of the proteasome inhibitor, bortezomib.

Flow Cytometry Data (Annexin V/PI)



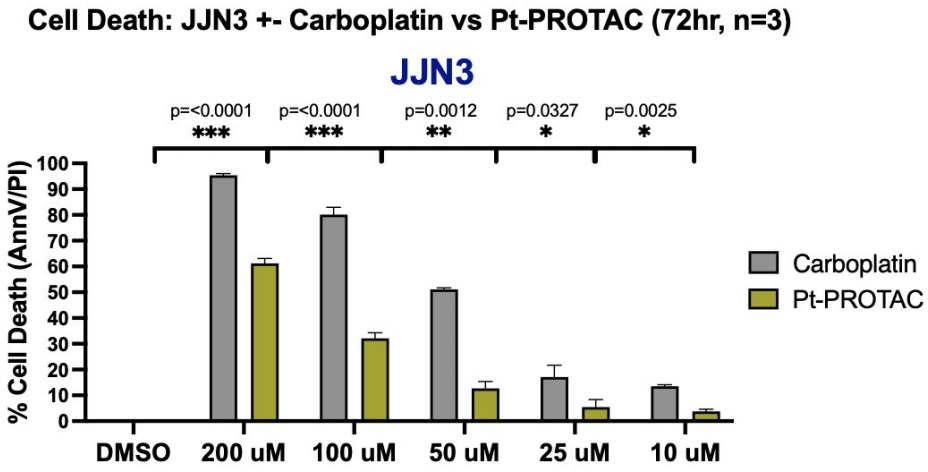
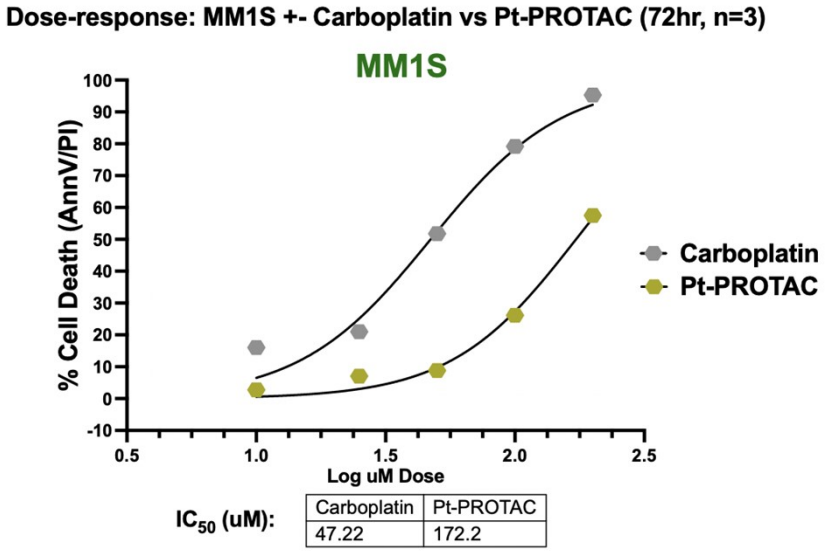


Figure S17: Dose-response plots for carboplatin and Pt-PROTAC against JJN3 cell line for 72 hrs (top) and percentage dead cell population following carboplatin and Pt-PROTAC treatment against JJN3 cell line for 72 hrs (bottom)



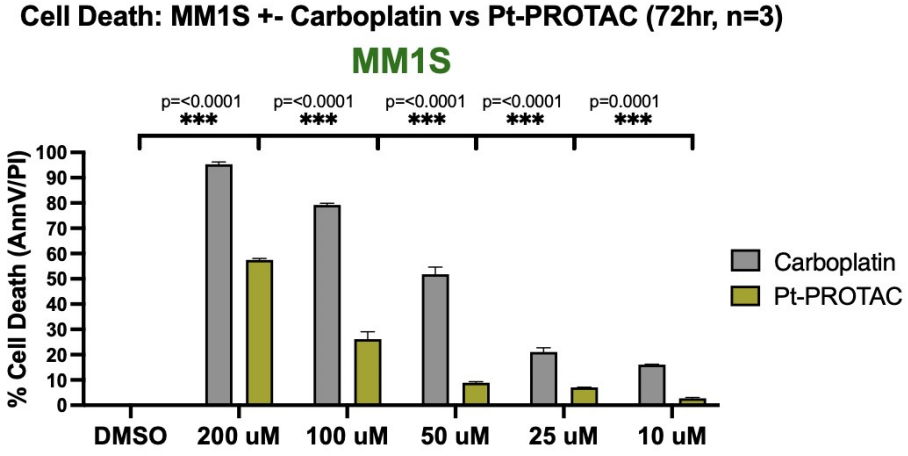


Figure S18: Dose-response plots for carboplatin and Pt-PROTAC against MM1.S cell line for 72 hrs (top) and percentage dead cell population following carboplatin and Pt-PROTAC treatment against MM1.S cell line for 72 hrs (bottom)

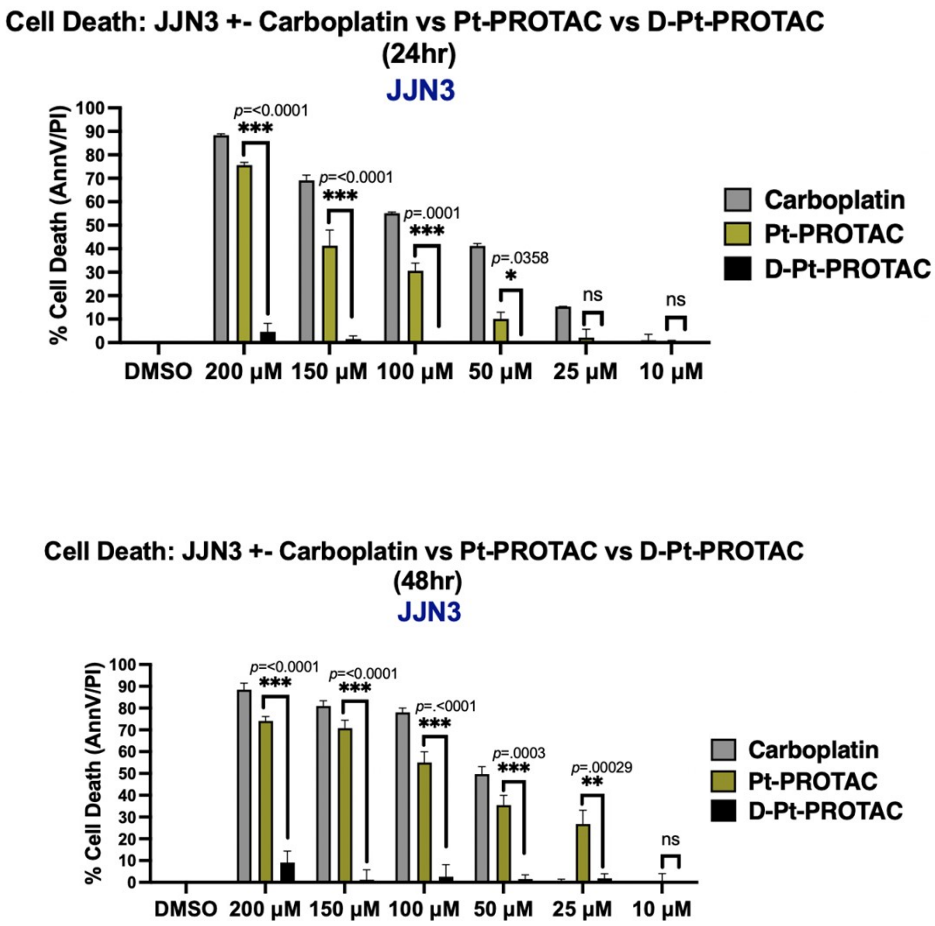


Figure S19: Percentage dead cell population following carboplatin and Pt-PROTAC treatment against JJN3 cell line for 24 and 48 hrs

References

1. Urankar, D. and J. Košmrlj, *Preparation of diazenecarboxamide–carboplatin conjugates by click chemistry.* Inorganica Chim. Acta, 2010. **363**(14): p. 3817-3822.
2. Wu, H., et al., *Development of Multifunctional Histone Deacetylase 6 Degraders with Potent Antimyeloma Activity.* J, Med. Chem., 2019. **62**(15): p. 7042-7057.