Supporting information

Re-evaluating the mechanism of action of α,β-unsaturated carbonyl DUB inhibitors b-AP15 and VLX1570: a paradigmatic example of unspecific protein crosslinking with Michael acceptor motif-containing drugs.

Jennifer A. Ward, [1,2,5‡*] Adan Pinto-Fernandez, [2,4 ‡*] Loïc Cornelissen, [3] Sarah Bonham, [2] Laura Díaz-Sáez, [1,2] Olivier Riant, [3] Kilian V. M. Huber, [1,2] Benedikt M. Kessler, [2] Olivier Feron, [4] and Edward W. Tate[5]

[1]Structural Genomics Consortium, Nuffield Department of Medicine, University of Oxford, Oxford, OX3 7DQ, UK.

[2]Target Discovery Institute, Nuffield Department of Medicine, University of Oxford, Oxford, OX3 7FZ, UK.

[3] Institute of Condensed Matter and Nanosciences, MOST division, Place Louis Pasteur, Université catholique de Louvain, Louvain-la-Neuve, B-1348, Belgium.

[4] Pole of Pharmacology and Therapeutics (FATH), Institut de Recherche Expérimentale et Clinique (IREC), UCLouvain, 57 Avenue Hippocrate B1.57.04, B-1200, Brussels, Belgium.

[5]Department of Chemistry, Imperial College London, Molecular Sciences Research Hub, White City Campus, London, W12 0BZ, UK.

‡JAW and APF contributed equally to this work.

*Corresponding authors: jennifer.ward@ndm.ox.ac.uk and adan.pintofernandez@ndm.ox.ac.uk

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Reagents

NeutrAvidin agarose resin and Dynabeads®MyOne™ Streptavidin C1 were purchased from Thermo Scientific. AzTB was synthesised as previously reported.[1]

Primary antibodies: anti-Ub (Cell Signaling; catalogue number 3936) anti-USP14 (Cell Signaling; catalogue number 11931), anti-UCH-37 (Abcam; catalogue number ab124931) anti-USP28 (Abcam, ab126604), anti-HA (12CA5) (Roche, 11583816001), anti-CIAPIN1 (Bethyl, A302-809A), anti-H3 (Cell signalling, 14269), anti-Actin (SantaCruz, sc-47778).

Cell culture

Cells were cultured at 37 °C in a humidified 5% CO_2 atmosphere in media (DMEM for HeLa, U2OS, and SiHa; RPMI for KMS11 and HCT116; EGM-2 for HUVECS) containing 10% FBS. Cells were grown until approximately 80% confluent before treatment with indicated concentrations of compounds or DMSO control.

Immunoblot analysis

Cells were washed with ice-cold PBS and lysed with RIPA buffer containing protease and phosphatase inhibitors. Proteins coming from either treated cells or treated cell extracts were separated by SDS-PAGE and detected with the indicated antibodies.

Ub-AML DUB activity Assay

DUB activities were measured using dedicated Glo Cell-Based Reagents and a Glomax microplate reader (Promega) following the manufacturer's instructions; for the DUB assay, Ub-AML (Boston Biochem, Cambridge, MA, USA) was used as a substrate. Activities of recombinant DUBs (purchased from Boston Biochem) or HeLa crude extracts (prepared as for ABP assays) were measured using this protocol.

Ha-UB-C2Br DUB ABP labelling assays

HA-Ub-C2Br was synthesised in the Kessler lab [2] and ABP assays were performed as previously described.[3] Briefly, Crude HeLa cell extracts were pre-incubated with DMSO (vehicle), b-AP15 (200 µM) and NEM (200 µM) for 30 minutes at 37°C, labeled with HAUbC2Br for 45 min at 37°C, separated in 4-15% SDS-PAGE gradient gels and immunoblotted with specific anti-USP28 antibody.

LC-MS analysis of GSH reaction with b-AP15

b-AP15 (10 µM) was incubated with GSH (1 mM) in DMSO for 30 minutes at room temperature, before analysis by LC-MS. Additional LC-MS analysis of b-AP15 and GSH alone were used as controls.

Compound 1 ABPP labelling assay

Cells were pre-incubated with indicated concentrations of VLX1570 or b-AP15 for 30 min before treatment with **1** for 1 hour. Cells were then lysed in Buffer C (1% v/v Triton X-100, 0.1% w/v SDS, 1x EDTA-free complete protease inhibitor (Roche Diagnostics) in PBS), protein concentrations were determined, and the lysates stored at -80 °C until use. Samples were analysed by in-gel fluorescent analysis following CuAAC ligation (see below).

CuAAC ligation

A premixed click cocktail (100 µM AzTb, 10 mM $CuSO_4$, 10 mM TCEP and 100 µM TBTA, final concentrations) was added to lysate adjusted to a final protein concentration of 1 mg/mL. The samples were vortexed for 1 h at room temperature and then quenched with 5 mM EDTA. Protein was precipitated by sequential addition of MeOH (2 × volumes), $CHCl_3$ (0.5 × volume) and H_2O (1× volume). Protein was pelleted (17,000 × g, 5 min) washed with MeOH (10 × volumes) and re-pelleted.

In-gel fluorescence

Pellets were air dried and suspended in PBS buffer containing 2% w/v SDS. 4× sample loading buffer (NuPAGE LDS sample buffer) containing 4% v/v β-mercaptoethanol was then added and samples heated for 6 min at 90 °C. Samples were separated by SDS-PAGE and the gels scanned using a Typhoon FLA 9500 Imager (GE Healthcare) equipped with 532 nm laser and LPG filter to visualise both the TAMRA fluorophore and molecular weight markers (Precision Plus All Blue Standards, Bio-Rad).

SILAC Cell Culture and Spike-in SILAC quantification

U2OS cells were cultured as previously described or in $^{15}N_4$ $^{13}C_6$-arginine and $^{15}N_2$ $^{13}C_6$-lysine (R10K8) containing DMEM media (Dundee Cell) with 10% v/v dialysed FBS. Cells grown in unlabelled media were treated in triplicate for 1 h with 5 µM of **1** after a 30 min pre-incubation with a fixed concentration of VLX1570 (0, 5 µM, 20 µM). In parallel, R10K8 incorporated U2OS cells were treated with 5 µM of **1** for 1 h to generate the 'spike-in' standard. Both spike and treatment cells were lysed in Buffer C, and stored at -80 °C until use.

Sample Preparation for Proteomic analysis

Sample preparation was conducted as previously described.[4] Briefly, 400 µg of each competition condition was mixed with 200 µg of spike, the protein concentration adjusted to 2 mg/mL, and CuAAC ligation performed. Protein pellets were resuspended in 0.2 % w/v SDS, 1 mM dithiothreitol in PBS to give a final protein concentration of 1 mg/mL. Samples were enriched on NeutrAvidin agarose resin (30 µL, pre-washed three times in 0.2% w/v SDS in PBS) by incubation with gentle shaking for 2 h at room temperature. The supernatant was then removed, and the beads washed consecutively with: 3 × 1% w/v SDS in PBS, 2 × 4M Urea in PBS, 5 × 50 mM ammonium bicarbonate (AMBIC). The washed beads were resuspended in 50 µL of 50 mM AMBIC and reduced with 10 mM dithiothreitol at 55 °C for 30 min. The samples were washed twice and resuspended in 50 µL of 50

mM AMBIC, and cysteines were alkylated by 10 mM iodoacetamide in the dark for 30 min. The samples were washed twice and resuspended in 50 µL of 50 mM AMBIC, treated with trypsin (5uL, 0.2 mg / ml, Promega) and digested overnight at 37 °C. The supernatant was retained and the beads washed with 80 µL AMBIC followed by 80 µL 0.1% v/v TFA in H2O (80 µL). The combined supernatants were desalted and dried in vacuo. Dried peptides were stored at -80 °C, and resuspended in 0.5% v/v TFA, 2% v/v MEOH in H2O (20 µL) for LC-MS/MS analysis.

ABPP LC-MS/MS data acquisition

LC-MS/MS runs were performed at Imperial College London on an Easy nLC-1000 system coupled to a QExactive mass spectrometer via an easy-spray source (all Thermo Fisher Scientific). 3 µL injections of peptide sample were separated on a reverse phase Acclaim PepMap RSLC column (50 cm x 75 µm inner diameter, Thermo Fisher Scientific) across a 2 h acetonitrile gradient containing 0.1 % v/v formic acid, using a flow rate of 250 nL/min. The instrument was operated in a data-dependent cycling mode with survey scans acquired at a resolution of 75,000 at m/z 200 (transient time 256 ms). The top 10 most abundant isotope patterns with charge +2 from this survey scan were then selected with an isolation window of 3.0 m/z and subjected to MS/MS fragmentation by HCD with normalized collision energies of 25. The maximum ion injection times for the survey scan and the MS/MS scans were 250 and 80 ms, respectively. The ion target value for MS was set to 106 and for MS/MS to 105, and the intensity threshold was set to 8.3 × 102.

ABPP Data analysis

The raw data was processed using MaxQuant version 1.5.0.253 and the reference complete human proteome FASTA file. 'Arg10' and 'Lys8' were selected as heavy labels, cysteine carbamidomethylation was selected as a fixed modification, and methionine oxidation as a variable modification. Default settings for identification and quantification were used. Specifically, a minimum peptide length of 7, a maximum of 2 missed cleavage sites, and a maximum of 3 labelled amino acids per peptide were employed. Peptides and proteins were identified utilising a 0.01 false discovery rate, with "Unique and razor peptides" mode selected for both identification and quantification of proteins (razor peptides are uniquely assigned to protein groups and not to individual proteins). At least 2 razor + unique peptides were required for valid quantification. Processed data was further analysed using Perseus version 1.5.0.9 and Microsoft Excel 2010. Peptides categorised by MaxQuant as 'potential contaminants', 'only identified by site' or 'reverse' were filtered, and the processed H/L ratios transformed in Log2(L/H) ratios. The ratios for each experimental condition were normalised relative to their median, and biological triplicates grouped. The detection of at least 2 unique peptides was used as a threshold for protein identification and 2 valid ratio values were required in at least one experimental group for quantification. Statistically significant competition was determined through the application of a P2 test, using a permutation-based FDR of 0.01 and an S0 of 0.1. To determine the relative response to inhibition, the average fold change was calculated for each protein under each inhibitor condition, by normalising all mean ratios relative to the condition lacking inhibitor (0 µM compound **1**).

GO term analysis of the 24 targets significantly competed by both 5 and 20 µM VLX1570 was conducted in STRING (http://string-db.org). Basic STRING settings were used for protein interaction network generation, with all active interaction sources (Textmining, Experiments, Databases, Co-

expression, Neighborhood, Gene Fusion, Co-occurrence) considered. The observed Gene Ontology Cellular component (GO CC) enrichment of this network is summarised in **Supplementary Table 1**.

Cell viability assays

KMS11 cells were seeded at 25,000 cells/well (100 µL) in a 96-well plate and treated with varying concentrations of VLX150 or compound **1**. For U2OS cells, cells were seeded at 10,000 (100 µL) in a 96-well plate and left overnight to adhere before continuing with the compound treatments described above. DMSO and bortezomib (10 mM) were used as positive and negative controls and all experiments were set up in biological triplicates. Following a 72 hour incubation, CellTiter Glo reagent was added according to manufacturer's instructions (Promega) and luminescence measured. EC50 values were determined by fitting the data to the IC_{50} dose response function using GraphPad Prism 7.

To analyse the impact of glutathione (GSH) on compound potency, KMS11 cells were seeded at 80,000 cell/well (100 µL) in a 96-well plate and treated with VLX1570 or b-AP15 (1 µM or 10 µM) with and without GSH present (1mM). DMSO was used as a control and all experiments were set up with 6 biological replicates. After 16 hours of incubation Alamar blue was added according to manufacturer's instructions (Thermo Scientific) with fluorescence measured at 560/590 nm excitation/emission. Cell viability was normalised to the DMSO control and the data plotted with Graphpad Prism 7.

siRNA studies

SMARTpool: ON-TARGETplus CIAPIN1 siRNA (Dharmacon, 4 µL, 10 µM stock solution) was added to 500 µL Opti-MEM in a 6-well plate and mixed gently before lipofectamine RNAiMAX (Thermo Fisher, 4 µL) was added. The mixture was incubated at room temperature for 20 minutes before addition of KMS11 cells (1.5 mL, 80,000 cells/mL) suspended in RPMI media. AllStars Negative Control siRNA (Qiagen) and water were used as scramble and vehicle controls, respectively, and all experiments were set up in biological triplicate. After 72 hours, 100 µL of cell suspension was transferred into a 96-well plate and cell viability measured by CellTiter Glo as described above. The remaining cells were pelleted and lysed as described above for immunoblot analysis.

Protein expression and purification

CIAPIN1 encoding pQTEV-LOC57019 was a gift from Konrad Buessow (Addgene plasmid # 34811; http://n2t.net/addgene:34811 ; RRID:Addgene_34811). pQTEV-LOC57019 was transformed into *E. coli* Rosetta cells. The cells were cultured in batch mode at 37 °C in the presence of 100 µg/mL of ampicillin and 34 µg/mL of chloroamphenicol in 100 mL TB (10 flasks in total) until an OD of 0.6 was reached. Batch mode was used, as degradation of the protein was observed under larger scale expression conditions. Induction was achieved with IPTG (200uM final concentration) and overnight incubation at 18 °C. The cells were harvested by centrifugation (4000 *g*, 30 minutes) and the resulting cell pellet lysed by sonication (15 sec on/off, 7.5 min) in 50 mL Buffer D (20 mM HEPES, 500 mM NaCl, 5% glycerol, 20 mM imidazole, 0.5% TCEP, pH 7.5) supplemented with Protease Inhibitor Cocktail Set III, EDTA-Free (Merck), and benzonase. The lysate was spun at 4000 *g* for 1 hour at 4 °C before the resulting supernatant was incubated for 45 minutes at 4 °C with HisPur™ Ni-NTA

superflow agarose beads (10 mL, Thermo Scientific) that had been pre-equilibrated in Buffer D (3 x 30 mL). The beads were then washed (5 x 10 mL Buffer D) and purified protein eluted with 50 mL Buffer E (20 mM HEPES, 500 mM NaCl, 5% glycerol, 500 mM imidazole, 0.5% TCEP, pH 7.5). The eluted protein was concentrated and further purified by size exclusion chromatography using a HiLoad 16/600 Superdex S200 collumn (GE Healthcare) equilibrated with Buffer F (20 mM HEPES, 150 mM NaCL, pH 7.5). The resulting protein purity was assessed by SDS-PAGE, and protein identity was confirmed by tryptic digest analysis (96% coverage). Protein concentration was determined to be 7.6 mg/mL, and was calculated using a theoretical molar extinction coefficient of 13,980 $M^{-1}cm^{-1}$ as determined by ProtParam.[5]

SDS page and Size exclusion analysis

Recombinant CIAPIN1 (10 µL, 1 mg/mL) was incubated with DMSO (0.16%) or VLX1570 at 1x, 2x, 5x, or 10x molar excess for 30 minutes at 4 °C before addition of sample loading buffer and analysis by SDS-PAGE.

For SEC analysis of aggregates, CIAPIN1 (100 µL, 7.6 mg/mL) was incubated DMSO (0.16%) or 2X molar excess of VLX1570 for 30 minutes at 4 °C before loading onto a Superdex S200 10/30 GL column (GE Healthcare) equilibrated with Buffer F. Gel filtration standard (BioRad) was used to generate a standard curve.

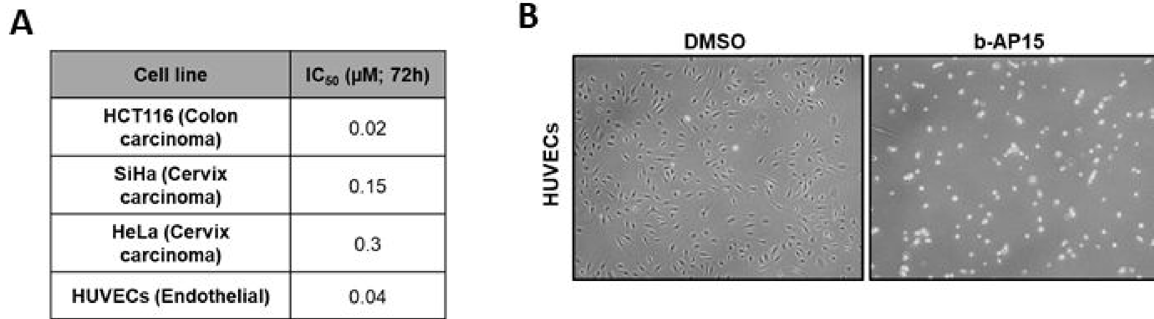
Peptide mapping analysis

Recombinant CIAPIN1 (5uL, 1mg/mL) was incubated with DMSO (0.16%) or VLX1570 (10x molar excess) for 30 minutes at 4 °C before Trypsin (Promega V5111, 2ul; 20ug in 100 ul Trypsin resuspension buffer) was added and the mixture incubated overnight at 37 °C. An aliquot of the digestion mixture (2 ul) was taken and diluted in 0.1% v/v FA, 2% v/v acetonitrile in H2O (30 µL) for LC-MS/MS analysis. Mass spectrometry data were acquired at the Discovery Proteomics Facility (University of Oxford). LC MS/MS analysis was performed using a Dionex Ultimate 3000 nano-ultra high pressure reverse phase chromatography coupled on-line to a Q Exactive mass spectrometer (Thermo Scientific). Samples were desalted online (PepMAP C18, 300µm x 5mm, 5µm particle, Thermo) for 1 minute at a flow rate of 20 ul/min and separated on an EASY-Spray PepMap RSLC C18 column (500 mm x 75 µm, 2µm particle size, Thermo Scientific) over a 60 minute gradient of 2-35 % acetonitrile in 5 % DMSO, 0.1% formic acid at 250 nl/min. MS1 scans were acquired at a resolution of 70,000 at 200 m/z and the top 15 most abundant precursor ions were selected for HCD fragmentation.
MS data were processed with PEAKS software (v 8.5),[6] searching against the UniprotKB Human database with protein of interest CIAPIN1 (Uniprot Q6FI81) included. Trypsin (specific, maximum 1 missed cleavage) was selected as the digestion enzyme. A mass tolerance of 10 ppm for precursor and 0.05 Da for fragment ions was used. Oxidation (M), Deamination (N, Q) and addition of VLX1570 (+469.11, single addition) were selected as variable modifications, and an FDR of 1% was applied for peptide matches.

ES-MS analysis

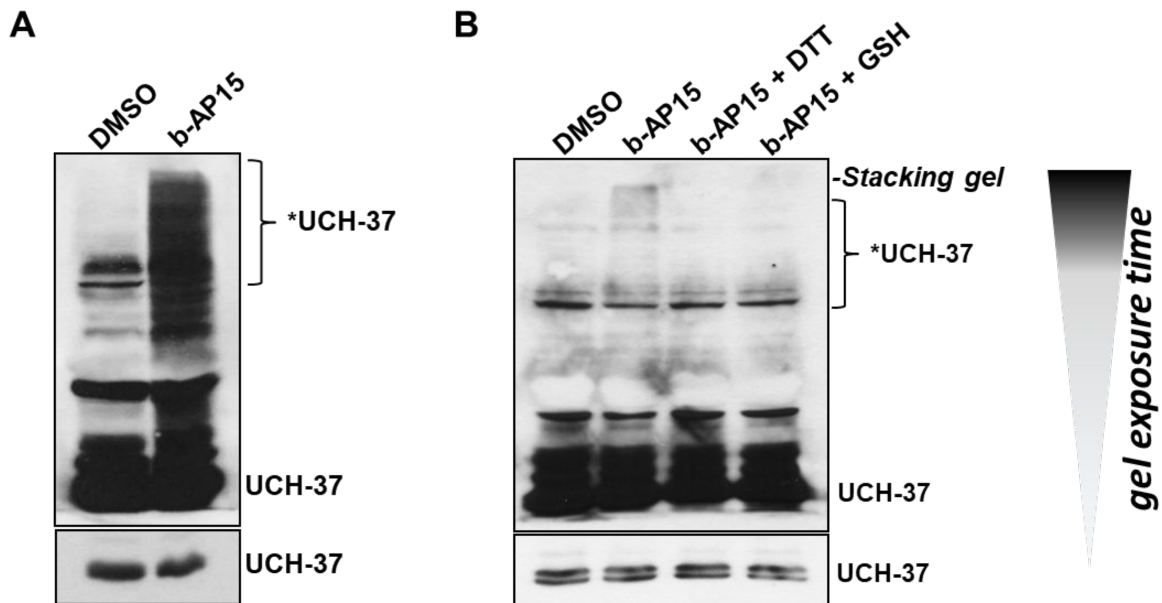
His-tagged CIAPIN1 (full length human (AA1-312) with a C-terminal 7x His tag and TEV site (KHHHHHHHS DYDIPTTENL YFQGS, 36502.38 Da, 1 mg/mL, 27 µM) was incubated with 10 molar equivalents of VLX1570 (270 µM) in PBS for 2 hours and analysed by ES-MS.

Supplementary Figures and Tables

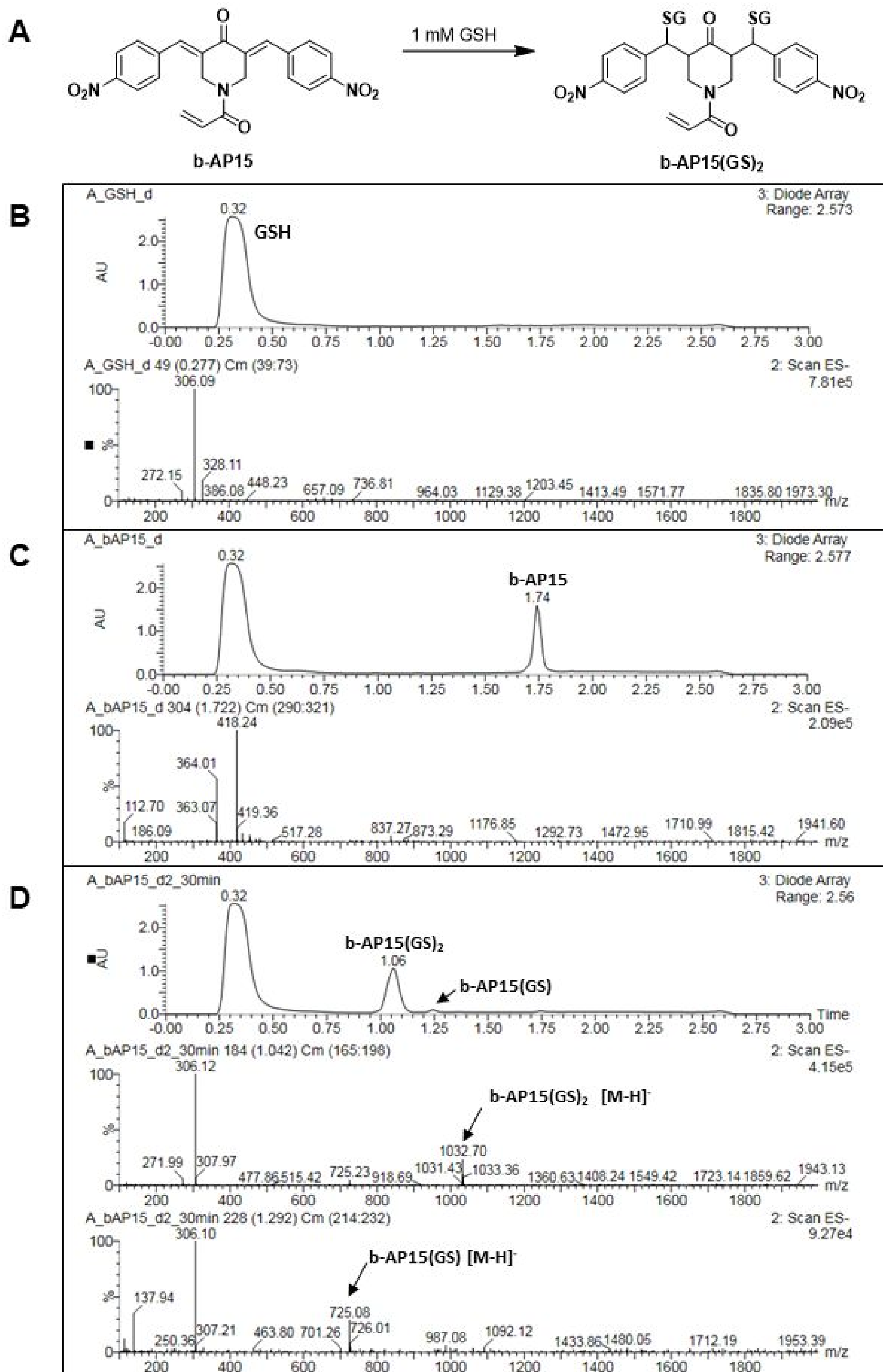


Cell line	IC_{50} (µM; 72h)
HCT116 (Colon carcinoma)	0.02
SiHa (Cervix carcinoma)	0.15
HeLa (Cervix carcinoma)	0.3
HUVECs (Endothelial)	0.04

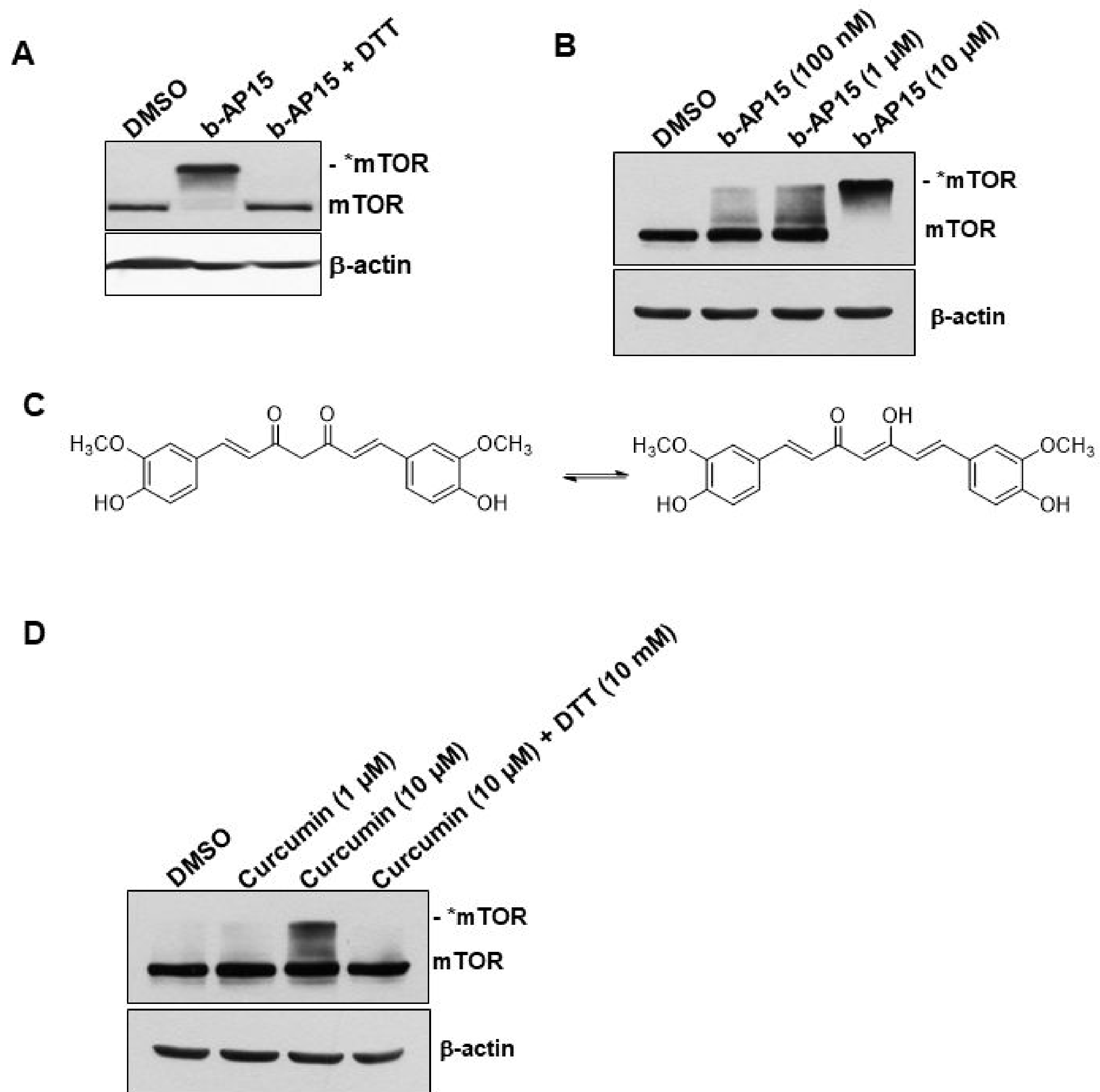
Supplementary Figure 1. (A) IC_{50} values measured by the CellTiter Glo assay for tumor and endothelial cell lines exposed to b-AP15 for 72 h (n=6). (**B**) Representative microscope picture showing the toxic effects of b-AP15 (1 µM) on HUVECs cells after 18 hours of incubation**.**



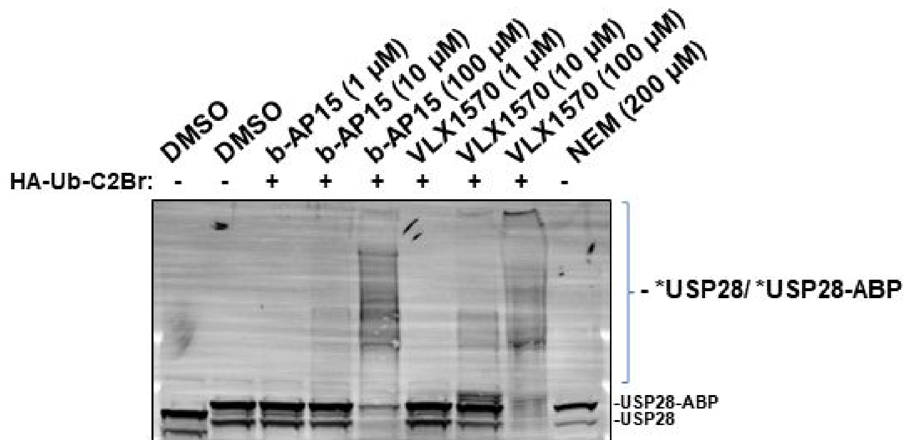
Supplementary Figure 2. Immunoblot analysis of high MW UCH-37 in (**A**) HeLa crude cell extracts incubated with b-AP15 (10 µM) for 2 hours at 37°C, and in (**B**) HeLa cells treated with either vehicle, 10 µM b-AP15 or with 10 µM b-AP15 in combination with reducing agents (10 mM dithiothreitol (DTT) or 10 mM glutathione (GSH)) for 2 hours. Shorter exposure times were used as a loading control (lower blots in the panel).



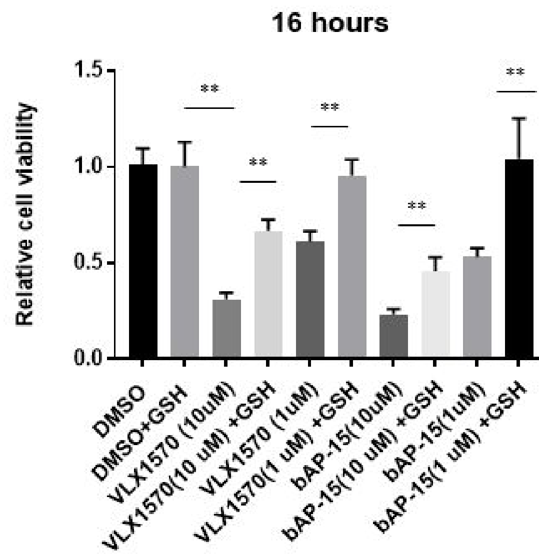
Supplementary Figure 3. (A) Schematic of GSH addition to b-AP15. Diode array chromatograms and ES^- spectra generated by LC-MS analysis of (B) GSH, (C) b-AP15 and (D) co-incubation of b-AP15 (10 μM) with GSH (1 mM) for 30 minutes.

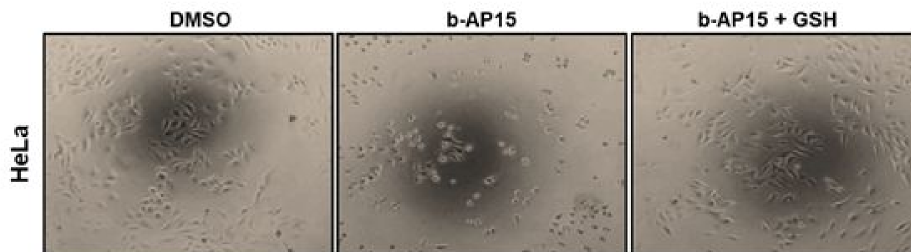


Supplementary Figure 4. Immunoblot analysis of high MW mTOR complex accumulation in **(A)** HeLa crude cell extracts incubated with b-AP15 (10 µM) with or without reducing agent for 2 hours at 37°C and **(B)** in HeLa crude cell extracts incubated with increasing concentrations of b-AP15 (100 nM, 1 µM and 10 µM) for 1 hour at 37°C. (**C**) Molecular structure of curcumin. (**D**) Immunoblot analysis of high MW mTOR complex accumulation in HeLa crude cell extracts incubated with curcumin or vehicle (DMSO) for 1 hour at 37°C, following a 15 minute pre-treatment with either vehicle or DTT (10 mM).

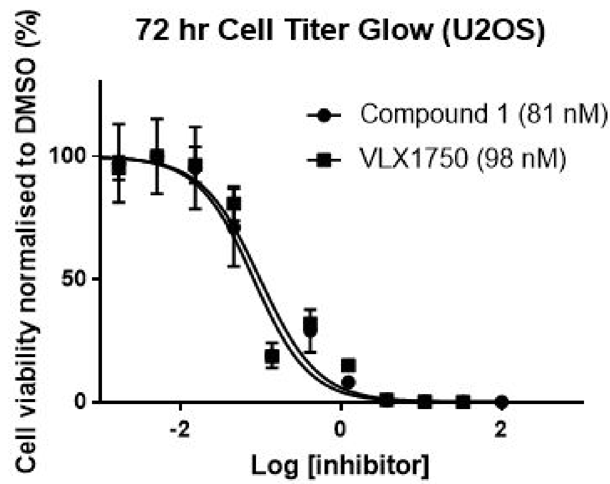


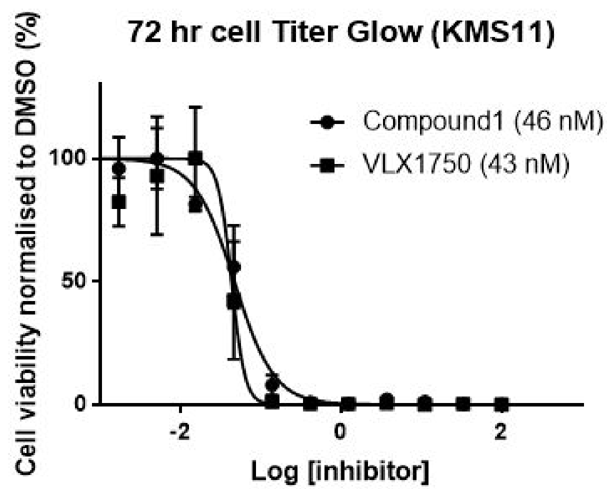
Supplementary Figure 5. Immunoblot analysis of high MW USP28 complex accumulation in crude HeLa cell extracts treated with DMSO, b-AP15, and NEM, for 30 minutes at 37°C and then labelled with HAUbC2Br.



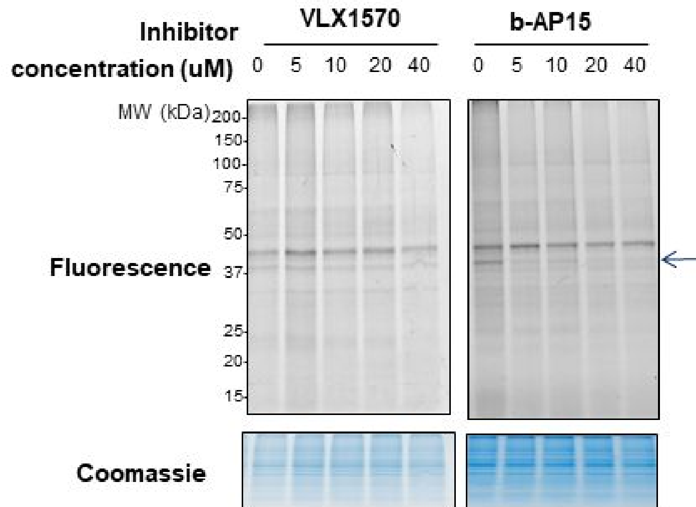


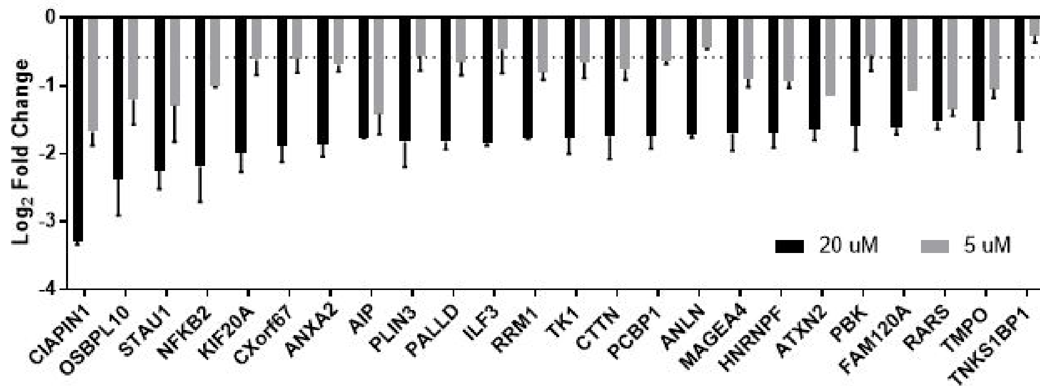
Supplementary Figure 6. (**A**) Alamar blue analysis of KMS11 cells incubated with compound with or without GSH (1 mM) for 16 hours (**B**) Representative microscope pictures of HeLa cells incubated with b-AP15 (10 µM) with or without reducing agents (10 mM) for 2 hours at 37°C.





Supplementary Figure 7. CellTiter Glo experiment with VLX1570 and compound **1** demonstrates conserved cytotoxicity in U2OS and KMS11 cells. IC_{50} values are given in parentheses.

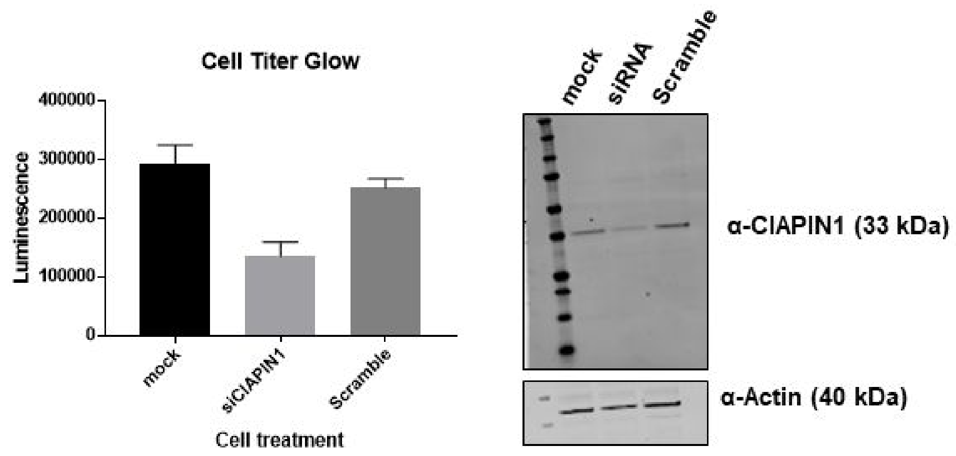




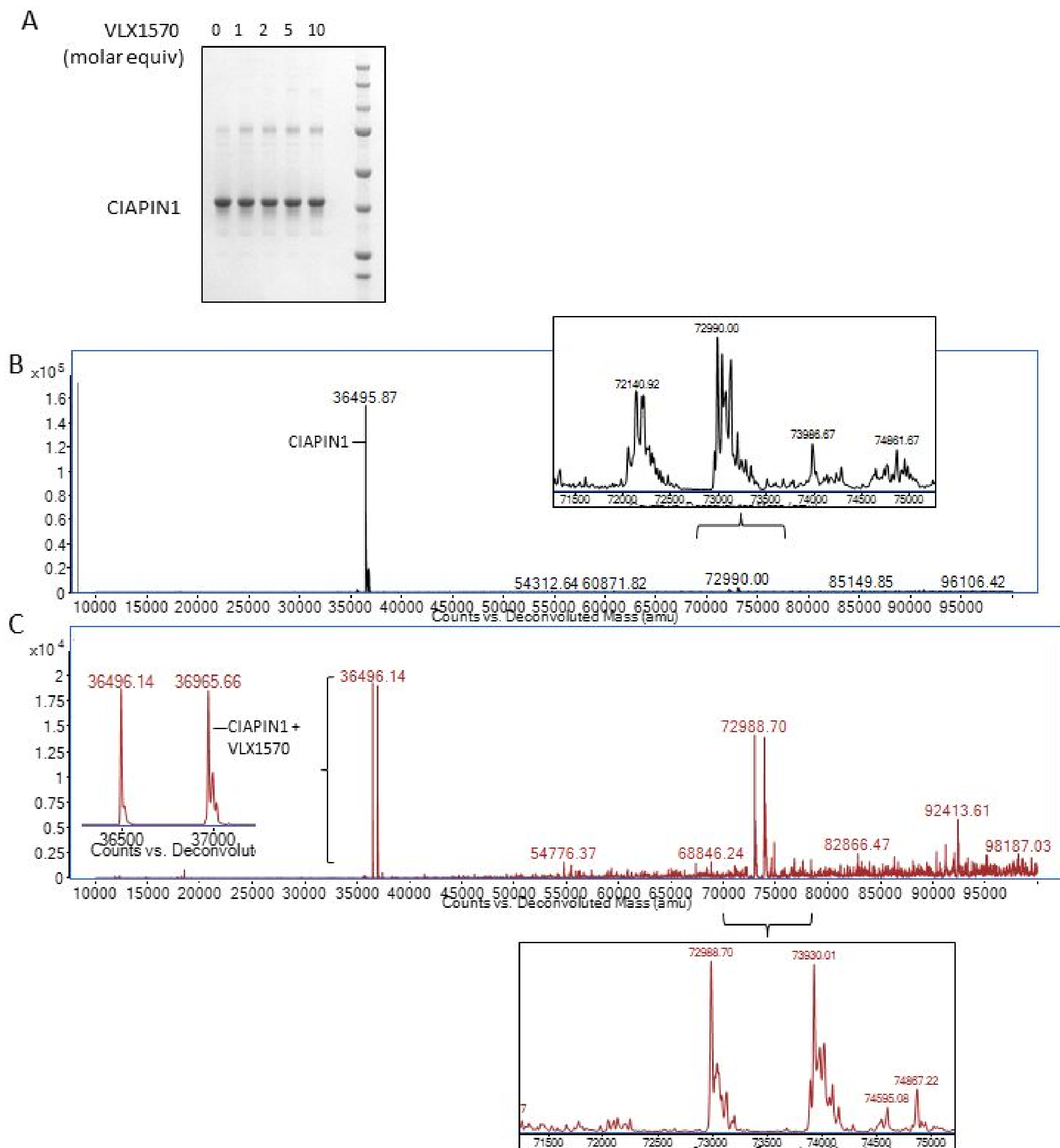
Supplementary Figure 8. (A) In-gel fluorescence analysis of compound **1** (5µM) labelled U2OS proteome in competition with VLX1570 and structurally related inhibitor bAP-15. **(B)** Targets of compound **1** (5 µM) identified as being significantly competed (FDR=0.05, S0 = 0.2) by VLX1570 at both 20 µM and 5 µM. The dashed line indicates a 1.5 fold change.

Supplementary Table 1. Gene Ontology Cellular Component (GO CC) enrichment analysis of the 24 targets competed by both 5 and 20 µM of VLX1570. Analysis was conducted using STRING software.

Pathway ID	pathway description	observed gene count	false discovery rate	matching proteins in your network (labels)
GO.0005829	cytosol	16	0.0053	AIP,ANXA2,ATXN2,CTTN,FAM120A,HNRNPF,NFKB2,OSBPL10,PALLD,PCBP1,PLIN3,RARS,RRM1,STAU1,TK1,TNKS1BP1
GO.0043232	intracellular non-membrane-bounded organelle	14	0.0059	ANLN,ANXA2,ATXN2,CIAPIN1,CTTN,ILF3,KIF20A,OSBPL10,PALLD,PCBP1,PLIN3,STAU1,TMPO,TNKS1BP1
GO.0002102	podosome	2	0.0207	CTTN,PALLD
GO.0032991	protein-containing complex	14	0.0207	AIP,ANXA2,ATXN2,CTTN,HNRNPF,ILF3,KIF20A,NFKB2,PALLD,PCBP1,RARS,RRM1,STAU1,TNKS1BP1
GO.0043231	intracellular membrane-bounded organelle	21	0.0207	AIP,ANLN,ANXA2,ATXN2,CIAPIN1,CTTN,CXorf67,FAM120A,HNRNPF,ILF3,KIF20A,NFKB2,PALLD,PBK,PCBP1,PLIN3,RARS,RRM1,STAU1,TMPO,TNKS1BP1
GO.0044428	nuclear part	13	0.0207	AIP,ANLN,CIAPIN1,CXorf67,HNRNPF,ILF3,KIF20A,NFKB2,PCBP1,RARS,RRM1,TMPO,TNKS1BP1
GO.0044444	cytoplasmic part	20	0.0207	AIP,ANLN,ANXA2,ATXN2,CIAPIN1,CTTN,FAM120A,HNRNPF,ILF3,KIF20A,NFKB2,OSBPL10,PALLD,PCBP1,PLIN3,RARS,RRM1,STAU1,TK1,TNKS1BP1
GO.0044446	intracellular organelle part	19	0.0207	AIP,ANLN,ANXA2,ATXN2,CIAPIN1,CTTN,CXorf67,HNRNPF,ILF3,KIF20A,NFKB2,PALLD,PCBP1,PLIN3,RARS,RRM1,STAU1,TMPO,TNKS1BP1
GO.0001726	ruffle	3	0.0232	ANXA2,CTTN,PALLD
GO.0030496	midbody	3	0.0232	ANLN,ANXA2,KIF20A
GO.0043229	intracellular organelle	22	0.0232	AIP,ANLN,ANXA2,ATXN2,CIAPIN1,CTTN,CXorf67,FAM120A,HNRNPF,ILF3,KIF20A,NFKB2,OSBPL10,PALLD,PBK,PCBP1,PLIN3,RARS,RRM1,STAU1,TMPO,TNKS1BP1
GO.0005634	nucleus	16	0.0255	AIP,ANLN,CIAPIN1,CXorf67,FAM120A,HNRNPF,ILF3,KIF20A,NFKB2,PALLD,PBK,PCBP1,RARS,RRM1,TMPO,TNKS1BP1
GO.0005737	cytoplasm	21	0.0255	AIP,ANLN,ANXA2,ATXN2,CIAPIN1,CTTN,FAM120A,HNRNPF,ILF3,KIF20A,NFKB2,OSBPL10,PALLD,PCBP1,PLIN3,RARS,RRM1,STAU1,TK1,TMPO,TNKS1BP1
GO.0010494	cytoplasmic stress granule	2	0.0255	ATXN2,STAU1
GO.0036464	cytoplasmic ribonucleoprotein granule	3	0.0255	ATXN2,PCBP1,STAU1
GO.1990904	ribonucleoprotein complex	5	0.0255	ATXN2,HNRNPF,ILF3,PCBP1,STAU1
GO.0005938	cell cortex	3	0.0262	ANLN,ANXA2,CTTN
GO.0032155	cell division site part	2	0.0262	ANLN,KIF20A
GO.0044424	intracellular part	23	0.0294	AIP,ANLN,ANXA2,ATXN2,CIAPIN1,CTTN,CXorf67,FAM120A,HNRNPF,ILF3,KIF20A,NFKB2,OSBPL10,PALLD,PBK,PCBP1,PLIN3,RARS,RRM1,STAU1,TK1,TMPO,TNKS1BP1
GO.0005811	lipid droplet	2	0.0321	ANXA2,PLIN3
GO.0030863	cortical cytoskeleton	2	0.0333	ANLN,CTTN
GO.0031981	nuclear lumen	11	0.0372	AIP,ANLN,CIAPIN1,CXorf67,HNRNPF,ILF3,KIF20A,NFKB2,PCBP1,RARS,TNKS1BP1
GO.0005654	nucleoplasm	10	0.0377	AIP,ANLN,CIAPIN1,CXorf67,HNRNPF,ILF3,KIF20A,NFKB2,PCBP1,RARS



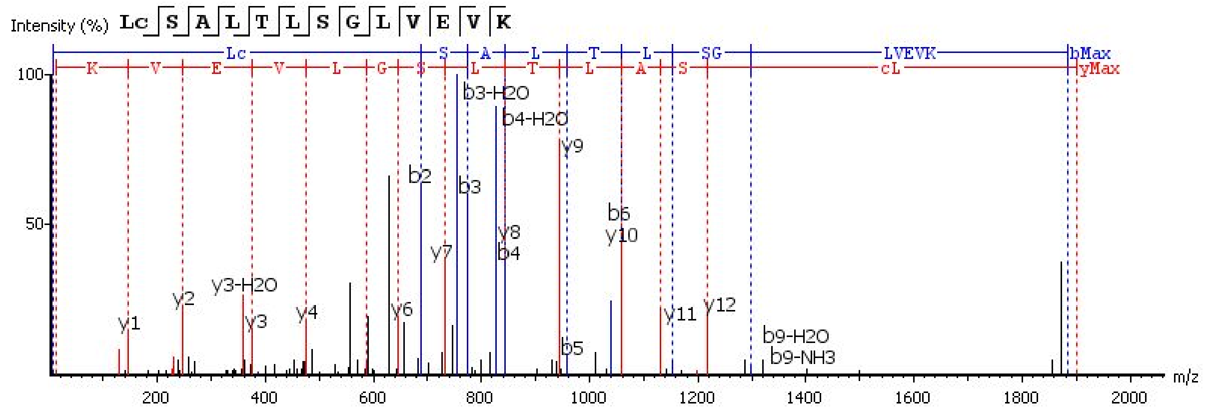
Supplementary Figure 9. CellTiter Glo analysis of cell viability and immunoblot analysis of CIAPIN1 knockdown efficiency following transfection of KMS11 cells with siRNA (CIAPIN1), Scramble or mock control.

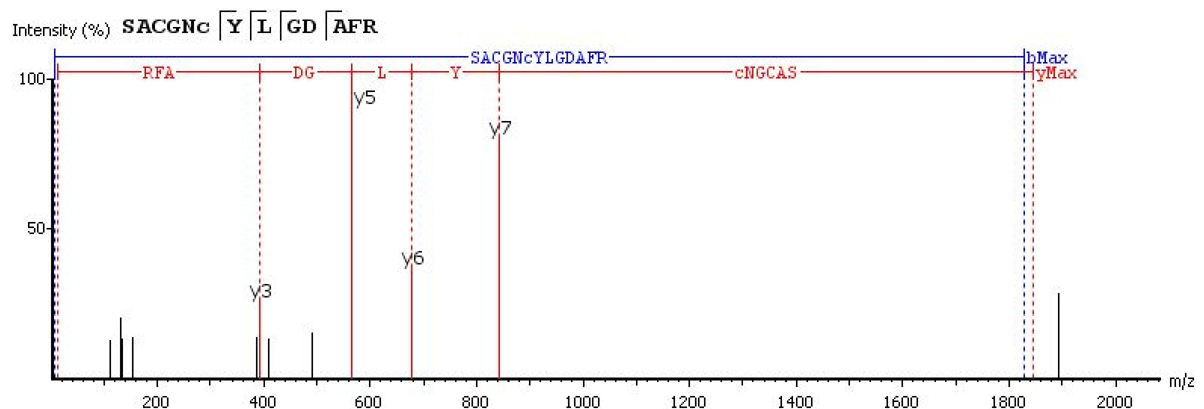


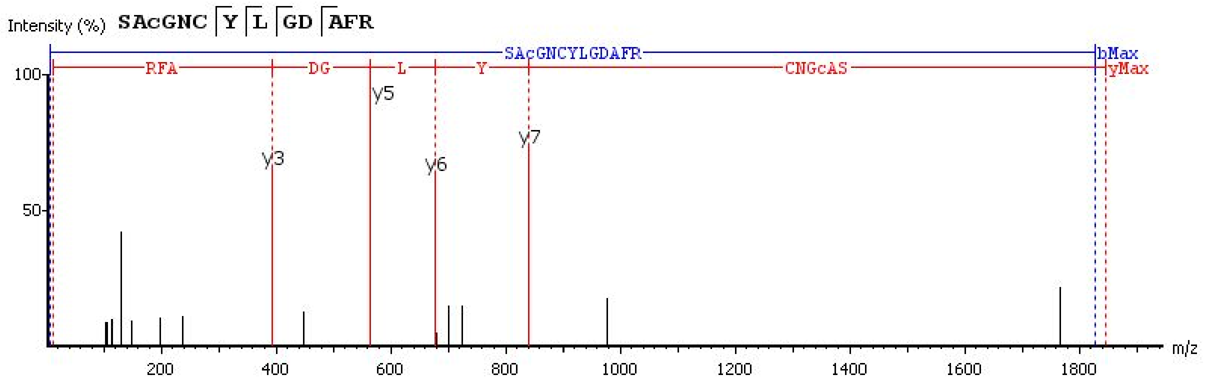
Supplementary Figure 10. (**A**) Coomassie staining of recombinant CIAPIN1 following incubation with indicated molar equivalent of VLX1570 or DMSO control. (**B**) ES+ of recombinant CIAPIN1 before and (**C**) after incubation with 10 molar equivalents of VLX1570 for 2 hours at room temperature confirmed the formation of a covalent adduct. Note: no peak corresponding to the covalent crosslinking of CIAPIN1 to give CIAPIN1-VLX1570-CIAPIN1 (73461 Da) was observed, suggesting that the high MW complexes of CIAPIN1 observed experimentally are formed non-covalently. Though peaks were observed at the masses corresponding to 2xCIAPIN (72990 Da) and 2xCIAPIN-VLX1570 (73930 Da) these are deconvolution artefacts, identified by their doubled Na+ adducts (+44 Da, rather than +22 Da, data not shown.)

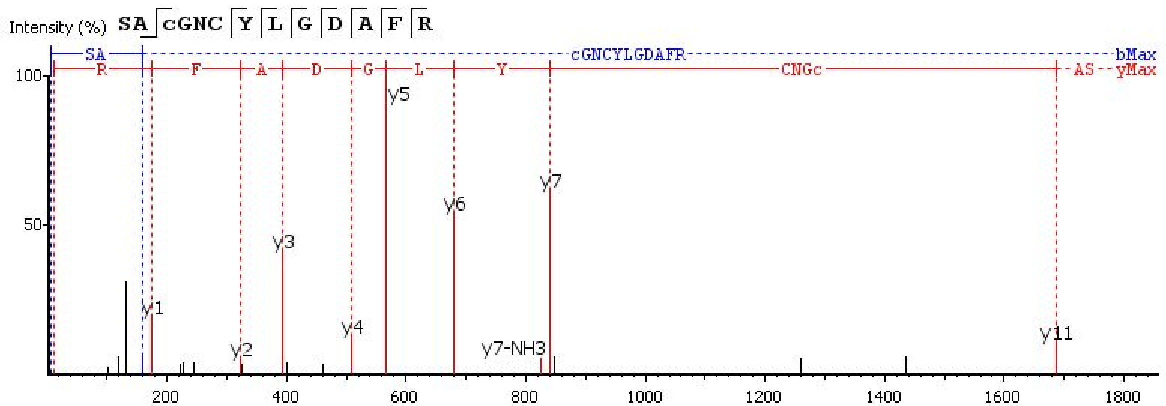
Supplementary Figure 11. Cysteine mapping of VLX1570 reveals 7 out of a possible 10 cysteine sites on CIAPIN1 are modified. Peptide coverage is 96%.

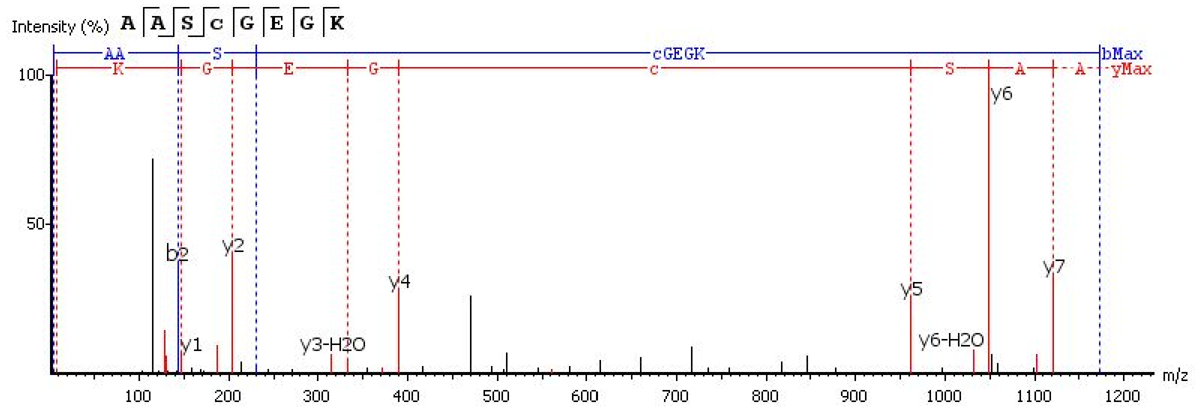
Supplementary Figure 12. Assigned spectra of VLX1570 modified peptides. Lower case represents a modified amino acid.

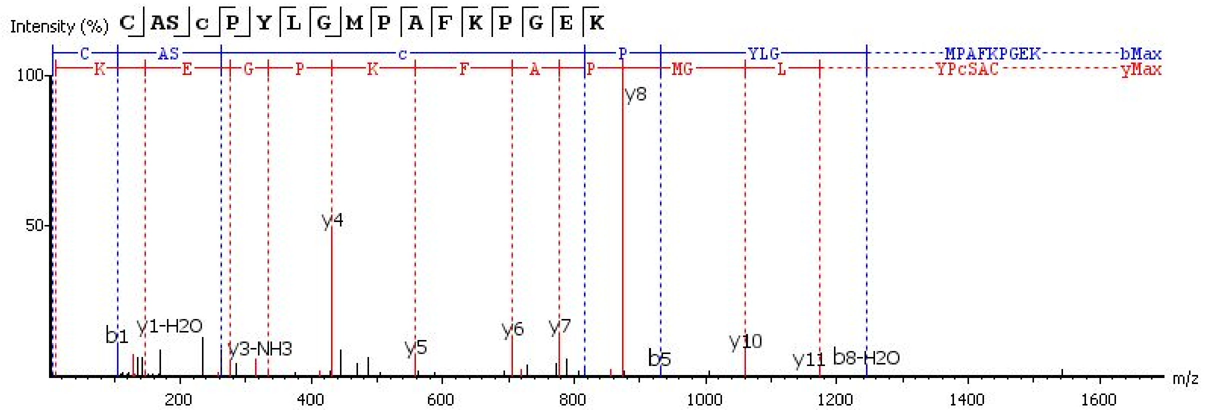


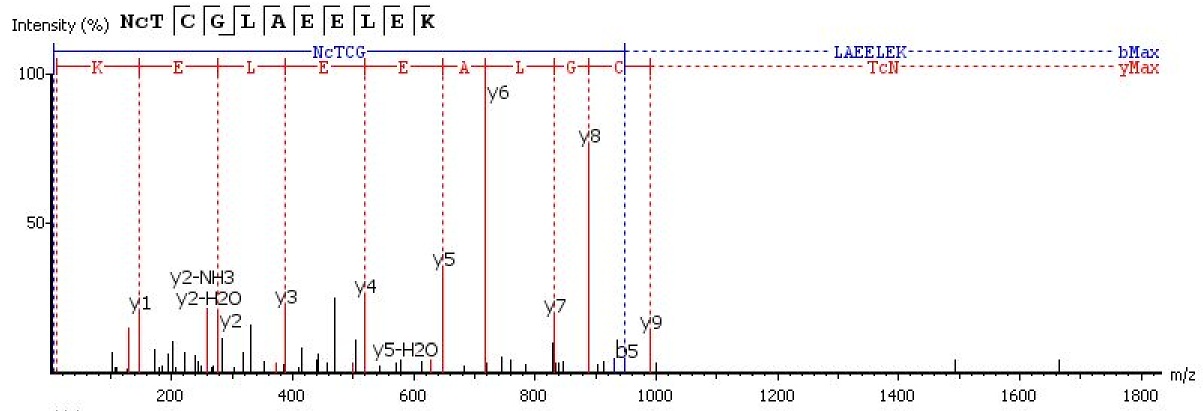


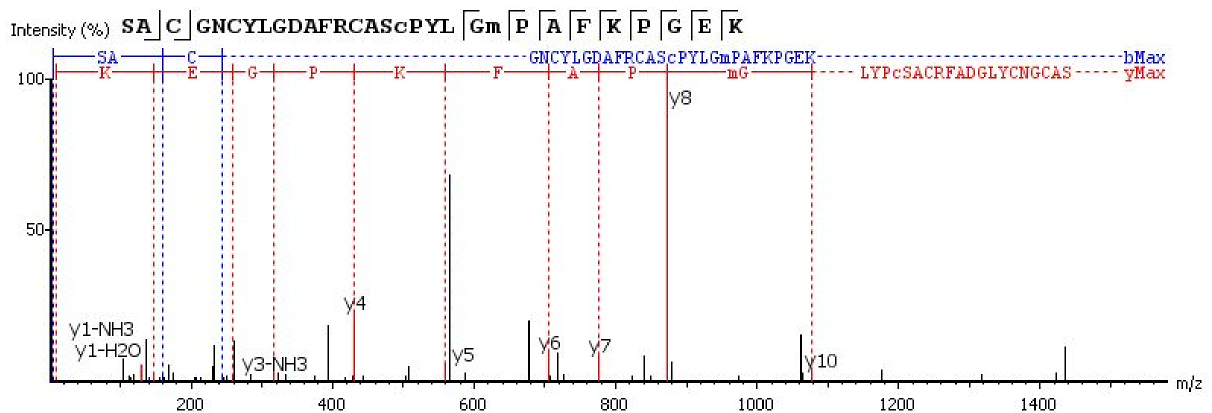





Intensity (%) A A S c G E G K
bMax
yMax
y6
b2
y2
y4
y5
y7
y1
y3-H2O
y6-H2O
m/z


Intensity (%) C AS c P Y L G M P A F K P G E K
bMax
yMax
y8
y4
b1
y1-H2O
y3-NH3
y5
y6
y7
b5
y10
y11
b8-H2O
m/z


Intensity (%) NcT C G L A E E L E K
bMax
yMax
y6
y8
y5
y2-NH3
y2-H2O
y1
y3
y4
y2
y7
y9
y5-H2O
b5
m/z


Intensity (%) SA C GNCYLGDAFRCAScPYL Gm P A F K P G E K
bMax
yMax
y8
y4
y1-NH3
y1-H2O
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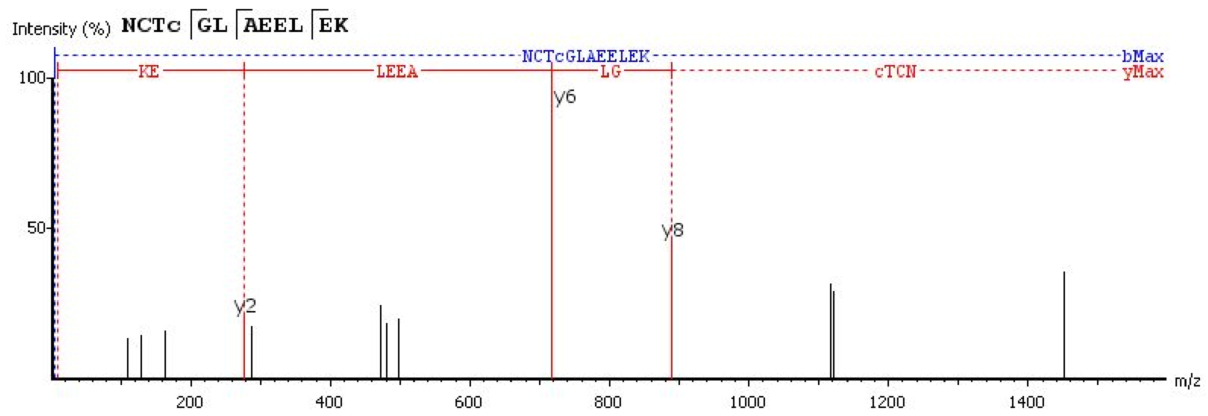
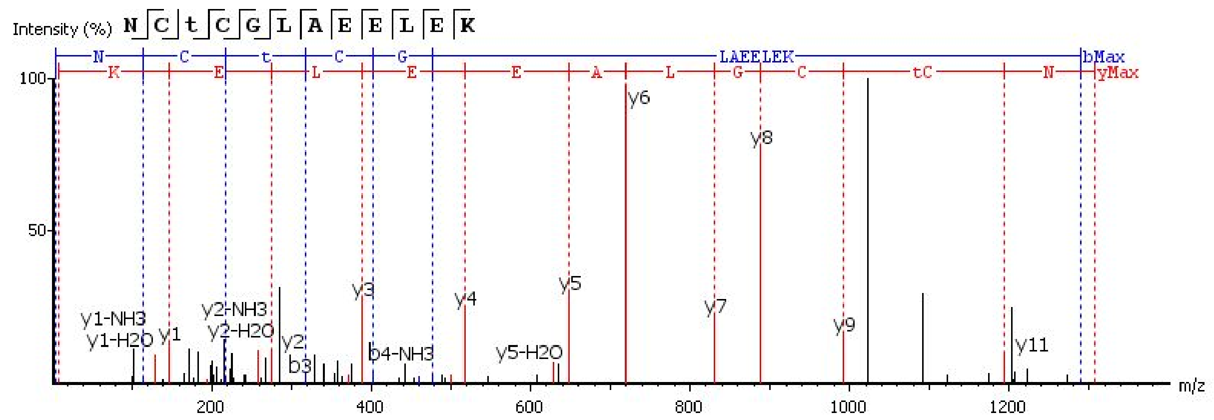
y6
y7
y10
m/z

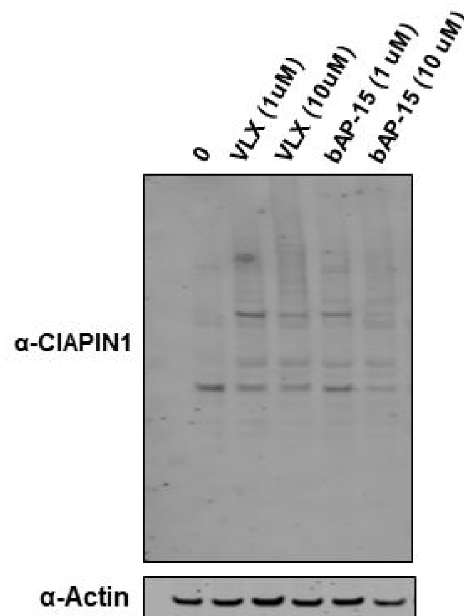


Unmodified Peptide comparison: (retaining disulfide bond)



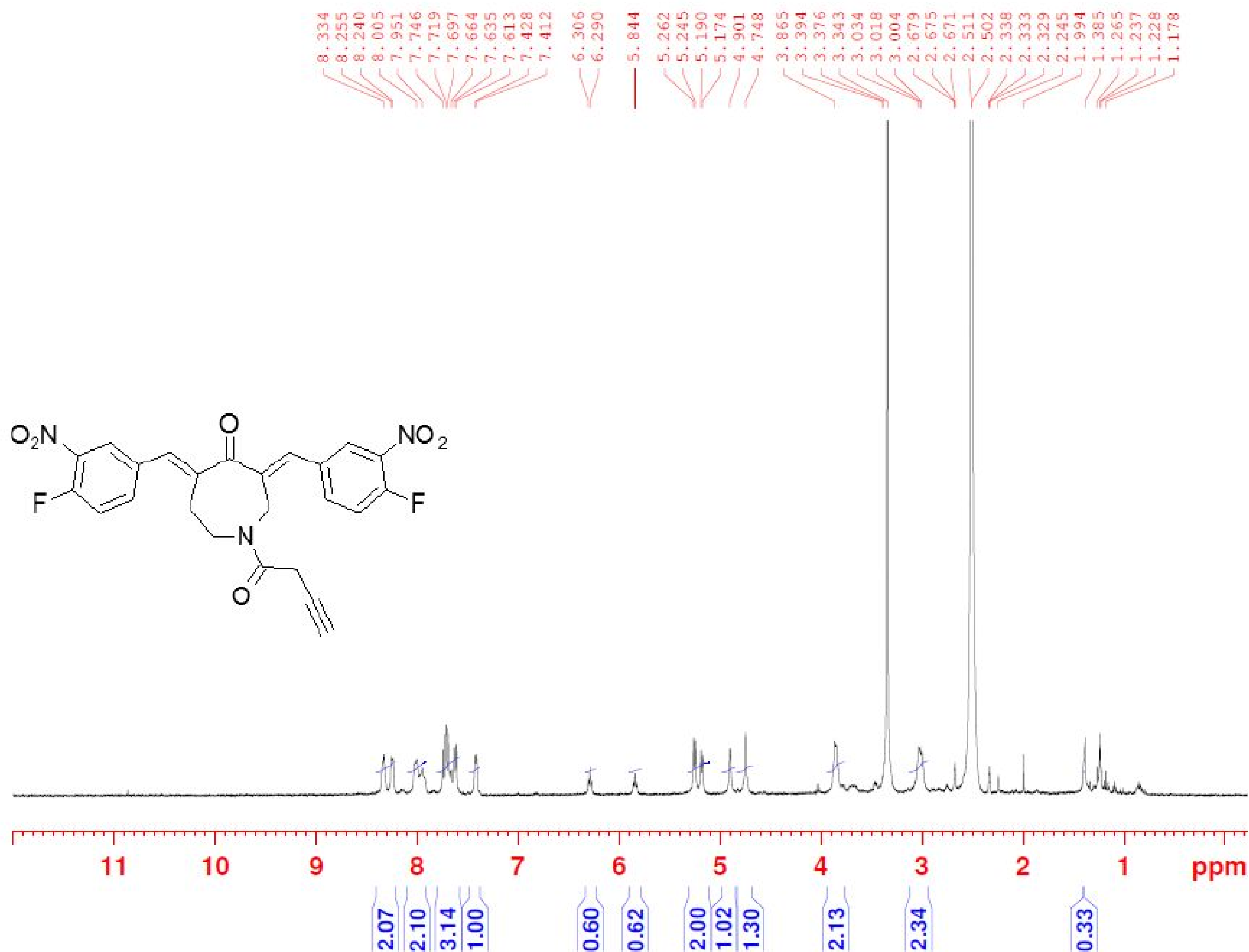
Supplementary Table 2. VLX1570-modified peptides identified by PEAKs analysis. Mass changes due to VLX1570 (+469.11), oxidation (+15.99) and disulfide bond (-2.02) formation are indicated. RT = retention time

Peptide Sequence	-10LogP	Mass	ppm	m/z	RT
LC(+469.11)SALTLSGLVEVK	74.76	1900.91	-0.6	951.4615	78.05
SACGNC(+469.11)YLGDAFR	69.08	1844.674	0.1	923.3443	74.62
SAC(+469.11)GNCYLGDAFR	66.97	1844.674	-0.3	923.3439	70.4
AASC(+469.11)GEGK	63.57	1190.416	0.3	596.2152	57.64
CASC(+469.11)PYLGMPAFKPGEK	60.5	2266.934	0.9	756.6526	63.21
NC(+469.11)TCGLAEELEK	43.58	1777.678	-0.2	889.8461	67.51
SACGNCYLGDAFRCASC(+469.11)PYLGM(+15.99)PAFKPGEK	33.63	3640.483	1.1	729.1047	56.76
NCTC(+469.11)GLAEELEK	32.71	1777.678	1.2	889.8473	68.89
NCT(-2.02)CGLAEELEK	56.56	1306.553	0.3	654.2841	41.46

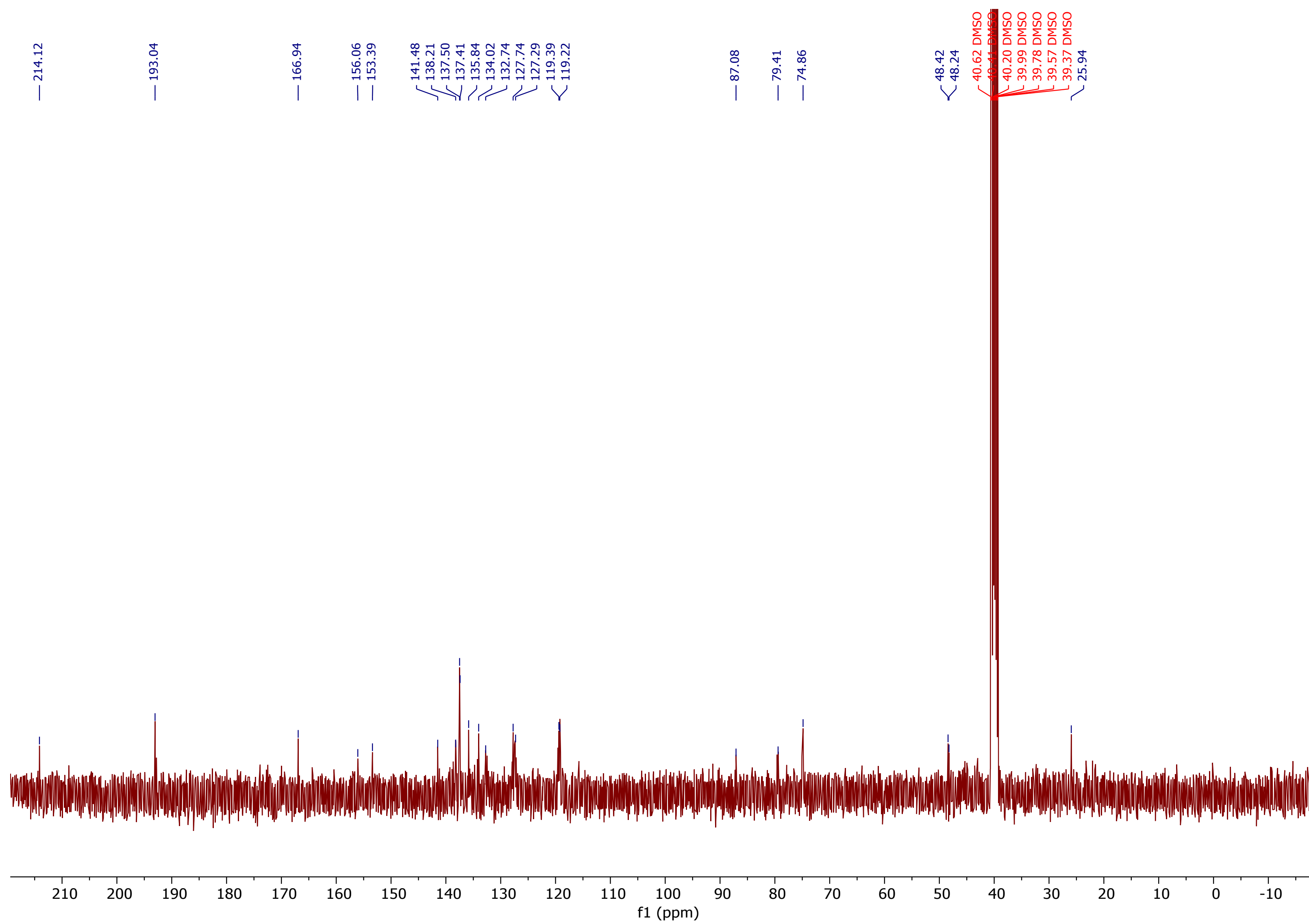


Supplementary Figure 13. Immunoblot analysis of CIAPIN1 following incubation with indicated concentrations of VLX1570 or b-AP15 for 6 hours demonstrated depletion of soluble CIAPIN1.

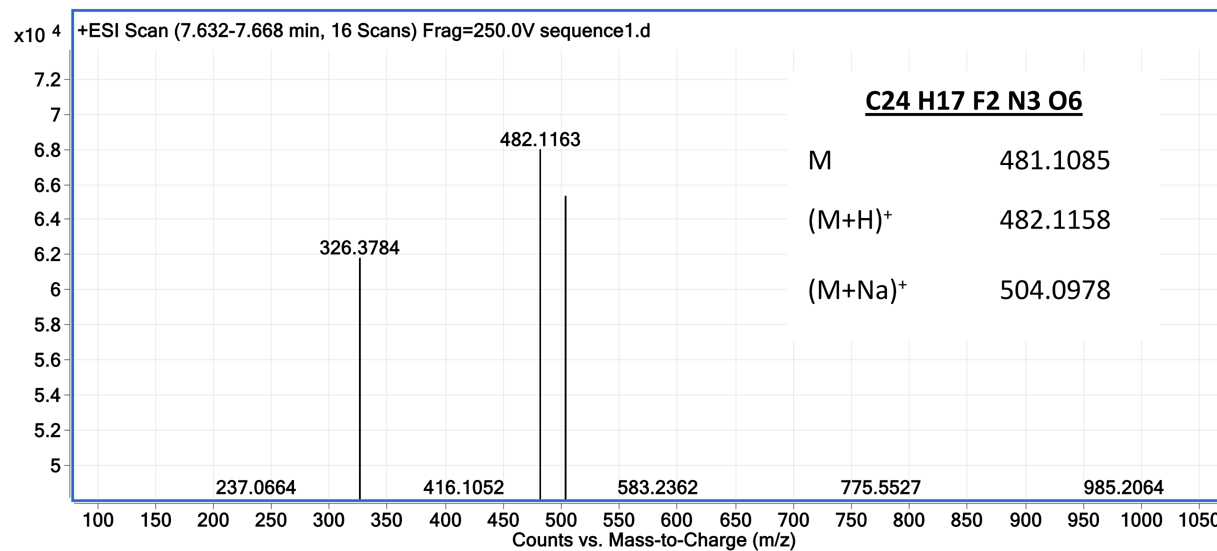
^{1}H NMR Spectra of Compound 1

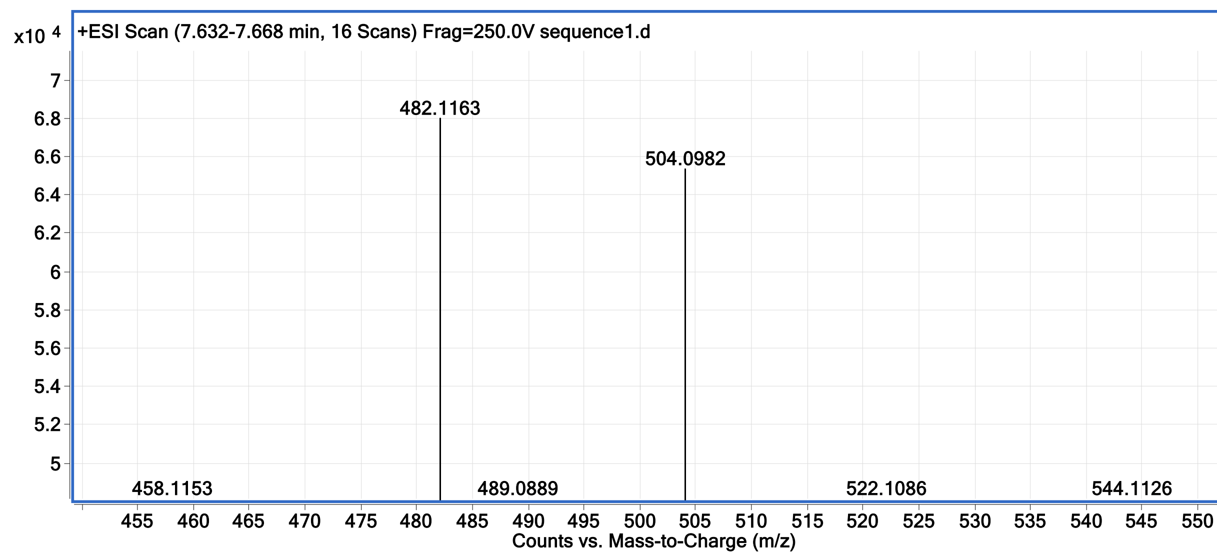


^{13}C NMR Spectra of Compound 1



HRMS Spectra of Compound 1





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