Supplementary Materials

Elucidation of the Hsp90 C-terminal Inhibitor Binding Site

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SUPPLEMENTAL MATERIALS AND METHODS

Identification of the Hsp90 C-terminal Protease Resistant Core

V8 digestion of Hsp90CT: Amino acids 531-732 from the C-terminal domain of human Hsp90a (Hsp90CT) was cloned into pProEX Hta plasmid. His-tagged human Hsp90α CT (containing an N-terminal vector derived leader of MSYYHHHHHHDYDIPTTENLYFQGAM-) was purified from BL21-Gold (DE3) cells (Invitrogen) by affinity chromatography using Ni-NTA resin (Qiagen) as described previously.(1) Hsp90CT (3.4 mg/ml) was dialyzed against 0.1 M sodium phosphate buffer (pH 7.8). Chlorobiocin (courtesy of the DrugSynthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, NIH) was dissolved in DMSO to a final concentration of 50 mM. V8 protease (SIGMA Cat No P2922) was reconstituted in 0.1 M sodium phosphate buffer pH 7.8 to a final concentration of 1 U/µl. Hsp90CT protein (250 µg) was incubated with 9 units of V8 protease at 37 ^{o}C in the presence of 800 µM Chlorobiocin or an equivalent volume of DMSO. Aliquots (50 µl) were collected at 0, 15 and 30 minutes of digestion, and immediately added to boiling SDS-PAGE sample buffer. Samples were run on a 15% SDS-PAGE gel, and polypeptides were visualized by staining with Commassie Blue. Polypeptide bands were excised, digested in-gel with trypsin, and analyzed as described below.

Trypsinolysis of V8 resistant peptide bands: Mass spectrometry analyses were performed in the DNA/Protein Resource Facility at Oklahoma State University, using resources supported by the NSF MRI and EPSCoR programs (award #0722494). MS-grade solvents were from Burdick and Jackson, or Baker. Sequencing grade trypsin was from Promega. Other solutions were the highest grade available from Sigma-Aldrich. Coomassie gel bands/spots were destained by extensive washing with 50% acetonitrile/50 mM ammonium bicarbonate pH 8, dehydrated with 100% acetonitrile, and dried briefly. Dried acrylamide pieces were rehydrated with 10 mM TCEP, 50 mM ammonium bicarbonate, and reduced for 1 H at room temperature. After incubation, the reducing buffer was replaced with 55 mM iodoacetamide in 50 mM ammonium bicarbonate, and alkylated for 1 hr at room temperature in the dark. Samples were then rinsed with ammonium bicarbonate, dehydrated with acetonitrile, and rehydrated/infiltrated with trypsin solution containing 8 µg trypsin per ml of 50 mM ammonium bicarbonate. After overnight digestion at 37 degrees C, the trypsinolytic peptide products were extracted with 0.5 % TFA, and used for subsequent analysis by mass spectrometry.

Methodology for LC-MS/MS analysis of peptides: Samples were analyzed on a hybrid LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific) coupled to a New Objectives PV-550 nanoelectrospray ion source and an Eksigent NanoLC-2D chromatography system. Peptides were analyzed by trapping on a 2.5 cm ProteoPrepII pre-column (New Objective) and analytical separation on a 75 µm I.D. fused silica column packed in house with10-cm of Magic C18 AQ, terminated with an integral fused silica emitter pulled in house. Peptides were eluted using a 5-40% ACN/0.1% formic acid gradient performed over 40 min at a flow rate of 300 nL/min.

During each one-second full-range FT-MS scan (nominal resolution of 60,000 FWHM, 300 to 2000 m/z), the three most intense ions were analyzed via MS/MS in the linear ion trap. MS/MS settings used a trigger threshold of 1000 counts, monoisotopic precursor selection (MIPS), and

rejection of parent ions that had unassigned charge states, were previously identified as contaminants on blank gradient runs, or were previously selected for MS/MS (data dependent acquisition using a dynamic exclusion for 150% of the observed chromatographic peak width). Column performance was monitored using trypsin autolysis fragments (m/z 421.76), and via blank injections between samples to assay for contamination.

Data analysis: Centroided ion masses were extracted using the extract_msn.exe utility from Bioworks 3.3.1 and were used for database searching with Mascot v2.2.04 (Matrix Science) and X! Tandem v2007.01.01.1 (www.thegpm.org). The following parameters were used: a parent ion mass tolerance of 15 ppm; a fragment ion tolerance of 0.8 Da; two missed tryptic cleavage; and variable modifications of pyroglutamate, oxidation of Met, alkylation by iodoacetamide and acrylamide adduct of cysteine.

Mascot was set up to search the IPI_Human_022209 database (75554 entries) assuming the digestion enzyme trypsin or semiTrypsin. X! Tandem was set up to search a subset of the IPI_Human_022209 database also assuming trypsin digestion. A database containing a single protein entry representing the sequence of the recombinant truncated His-tagged Hsp90 construct was used for the analysis cleavages at the N-terminus of the recombinant protein, the sequence of which is not present in native Hsp90. Peptide and protein identifications were validated using Scaffold v2.2.00 (Proteome Software) and the PeptideProphet algorithm.(2) Probability thresholds were greater than 99 % probability for protein identifications, based upon at least 2 peptides, identified 80 % certainty. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Limiting peptides generated from the N-terminus of Hsp90CT by V8/trypsin and trypsin/trypsin cuts or generated from the C-terminus by trypsin//V8 and trypsin/trypsin cuts were used to identify the chlorobiocin-protected protease resistant core.

Identification of the NB binding site in Hsp90's C-terminal Domain

Analysis of novobiocin fragmentation: Fragmentation spectra of unmodified novobiocin (Sigma N1628) were collected manually using an LTQ Orbitrap XL mass spectrometer. Novobiocin was dissolved to 5 mM in 50% acetonitrile/50% water/0.1% formic acid, and infused directly into ion source using 4 micron metal-coated glass infusion tips (New Objective). Individual ions were fragmented by CID in the ion trap, using collision energies of 35 eV (MS2 of 613 ion) and 40 eV and 25 eV for fragmentation of the 613 and 389 ions, respectively, for MS3. MS/MS and MS3 fragments thus produced were analyzed using the LTQ ion trap mass analyzer (profile mode).

Photoaffinity crosslinking of Hsp90CT: Amino acids 531- 732 of human Hsp90α expressed from the pQE32 plasmid was purified from BL21(DE3) as described previously.(1) To assess the ability of photo-affinity analogs to bind Hsp90CT, 50 µl of Hsp90CT (0.8 mg/ml) in 20 mM Tris-HCl (pH 7.4) and 50 mM NaCl was incubated with 5 mM **1** (**Fig. 3**) or vehicle control on ice for 30 min. Aliqouts (10 µl) were then added to 25 µl of 10 mM Tris-HCl (pH.7.4) containing 150 mM NaCl, 4 mM $CaCl_2$, 0.1 mM EDTA and trypsin as indicated in the figure legend (Supplemetary Figure 1C). Samples were then incubated for 6 min on ice followed by

addition of boiling SDS-PAGE sample buffer and analysis of the samples by SDS-PAGE on 12% gels.

For optimizing the photo-crosslinking reaction, Hsp90CT (25 µg) or myoglobin was incubated in buffer alone (10 mM Tris-HCl pH 7.4, 1500 mM NaCl , 4 mM $CaCl_2$, 0.1 mM EDTA) or buffer containing 5 mM **1** at 20 ^oC for 10 min. Samples were then spotted onto a microscope slide and placed 7 cm below the UV-bulb (254 nm) in a Stratagene UV Stratalinker 1800 followed by exposure to four flashes of 184 µJ of UV radiation. MALDI-TOF samples were prepared by diluting aliquots (1.5 µl) with 8 volumes of saturated Sinapinic acid matrix in 30% acetonitrile and 0.1% triflouroacetic acid, and were spotted onto a MALDI plate and allowed to dry. MS spectra were acquired in the positive linear mode (25,000 V accelerating voltage, 91% grid voltage, 1000 Da low mass gate) with 25 laser shots per spectrum using an Applied Biosystems Vayager-DE Pro MALDI-TOF.

To identify the site of crosslinking, recombinant Hsp90-CT (30 µg) was mixed with 0.5 mM of either **1**, **2** or DMSO (vehicle control) in buffer containing 10 mM Tris-HCl (pH7.4) 150 mM NaCl, 4 mM $CaCl_2$ and 0.1 mM EDTA. The reactions were incubated in the dark at room temperature for 30 minutes. Then each reaction was UV irradiated as described above. To confirm crosslinking, an aliquot of each was taken for analysis by MALDI-TOF MS as described above. Trypsin proteolysis was done by incubating each sample with 16 µg/ml trypsin at 37°C overnight.

Capillary liquid chromatography-mass spectrometry (LC-MS/MS) analysis: LC-MS/MS analysis was carried out at the University of Kansas Mass Spectrometry facility. Tryptic peptides from control and crosslinked samples were subjected to capillary LC-MS/MS experiments using tandem LTQ-FT Mass Spectrometer (ThermoFinnigan) under conditions described previously.(3) In short, peptides were separated on a reverse-phase *LC Packings PepMap* C-18 column (0.300 × 150 mm) at a flow rate 10 µl/min with linear gradient rising from 0 to 65% acetonitrile in 0.06% aqueous formic acid over a period of 55 min using LC Packing Ultimate Chromatograph (Dionex). LC-MS experiments were performed in a data-dependent acquisition manner using Xcalibur 1.4 software (Thermo Scientific). The five most intensive precursor ions in a survey MS1 mass spectrum acquired in the Fourier transform-ion cyclotron resonance over a mass range of 300–2000 *m/z* were selected and fragmented in the linear ion trap by collision-induced dissociation. The ion selection threshold was 1000 counts. Raw experimental files were processed by TurboSequest search using BioWorks 2.0 software. The resulting MS/MS peak lists in data format were combined within each experiment using an in-house written Perl script and submitted for peptide/protein identification to Mascot (Matrix Science, version 2.2) database-searching program using a custom database with a fragment ion mass tolerance of 0.20 u and a parent ion tolerance of 1.2 u to account for higher isotopes. A delta mass of 527 u was considered as variable modification on lysine residues.

Sequest and Mascot results were imported into Scaffold software (Proteome Software Inc., version 1.0) for analyzing with X!Tandem search algorithm and statistical validation of peptide/protein identities. Peptides validated by Scaffold at a confidence level of 50% and greater assigned by the Protein Prophet algorithm were taken to calculate protein coverage.(4) Identification of peptides at a confidence level of less than 50% was considered positive if their

corresponding m/z value from the survey MS1 scans were found to be within 10 ppm mass tolerance. Additionally, MS3 spectra were acquired for the most intensive ions from MS2 spectra that were identified as potential candidates containing the target modification. Fragmentation patterns of crosslinked peptides were analyzed using Protein Prospector v5.6.0 using a 0.2 Da error for peak matching.

Modeling of the Hsp90 NB binding site.

Molecular dynamics for model refinement: Following the initial 20000 minimization steps described under "Methods" in the manuscript, equilibration was done in steps by gradually increasing the system temperature in steps of 20K, from 10K to 310K and at each temperature a 15000 step (30 ps) equilibration was run keeping a restraint of 10 Kcal/mol$Å^2$ on protein alpha carbons. Thereafter, the system was equilibrated for 150000 steps (300ps) at 310K (NVT) and then for an additional 150000 steps (300ps) at 310K using Langevin piston (NPT) to achieve uniform pressure. Finally the restrains were removed and the system was equilibrated for 500000 steps (1ns) to prepare the system for simulation.

An NPT simulation was run on the equilibrated structure for 1 ns keeping the temp at 310 K and pressure at 1 bar using Langevin piston coupling algorithm. The integration time step of the simulations was set to 2.0 fs, the SHAKE algorithm was used to constrain the lengths of all chemical bonds involving hydrogen atoms at their equilibrium values and the water geometry was restrained rigid by using the SETTLE algorithm. Nonbonded van der Waals interactions were treated by using a switching function at 10Å and reaching zero at a distance of 12Å. The particle-mesh Ewald algorithm (PME) as implied in NAMD(5) was used to handle long range electrostatic forces.

Homology modeling and refinement of the structure of the NB binding site in Hsp90α**:** Homology modeling was performed with the program Modeller[6] with the structure of HtpG generated as above as a template (6). The sequence alignment was done as discussed previously and ten models were built followed by refinement using molecular dynamics as implied in Modeller (refine_fast). The model corresponding to the lowest value of the probability density function and fewest restraints violations out of the generated models was used for further analysis. The refinement of Hsp90α was carried out as described for HtpG. The length of the simulation was however short at 100ps.

SUPPLEMENTARY TABLES

Supplementary Table 1. Ion assignments for the MS2 spectrum of the unmodified (A), **2** crosslinked (B) and **1b** crosslinked peptides (C).

A. Ion assignments for the MS2 spectrum of the unmodified MH^{1+} ion 789.45 (KKQEEK).

Parental Ion (m/z)	Fragment (m/z)	Charge	Intensity	Ladder Ion	Neutral Loss H_2O / NH_3	R
789.45		+1		MH		H
	772.54	+1	1378	MH	$-NH_3$	H
	771.50	+1	6088	MH	$-1H_20$	H
	661.41	+1	345	y5 or (b5 + H_20)		H
	643.35	+1	247	b5 or (y5 - H_20)		H
	626.44	+1	54	b5	$-NH_3$	H
	625.30	+1	205	b5	$-1H_20$	H
	607.98	+2	98	b5	$-2H_20$	H
	533.36	+1	175	y4		H
	515.37	+1	88	y4	$-1H_20$	H
	514.30	+1	63	b4		H
	497.37	+1	51	b4	$-NH_3$	H
	496.27	+1	94	b4	$-1H_20$	H
	486.32	+1	18	a4		H
	385.35	+1	35	b3		H
	368.30	+1	48	b3	$-NH_3$	H
	257.17	+1	76	b2		H

B. Ion assignments for the MS2 spectrum of MH^{2+} 658.80 and MH^{1+} 1316.60 ions of the **2** crosslinked peptide (KKQEEK).

Parental Ion (m/z)	Fragment (m/z)	Charge	Intensity	Ladder Ion	Neutral Loss H_2O / NH_3	R
658.80		+2		MH		R1
	1100.45	+1	3648	b5	$-2H_20/-2NH_3$	R1
	1099.47	+1	13049	MH		R2
	824.45	+1	20	b4		R2
	764.36	+1	215	y5 or (b5 + H_20)		R3
	718.36	+1	837	a5		R3
	599.27	+1	530	b4	$-1H_20$	R3
	550.44	+2	766	MH		R2
	446.70	+2	286	MH		R3
	389.37	+1	20	z3		
	218.12	+1	330	Noviose		
1316.60		+1		MH		R1

	1299.61	+1	146	MH	-1NH_3	R1
	1298.80	+1	95	MH	-1H_20	R1
	1170.42	+1	60	b5		R1
	1100.45	+1	262	b5	-2H_20/-2NH_3	R1
	1099.48	+1	2127	MH		R2
	1082.50	+1	105	MH	-1NH_3	R2
	1081.56	+1	57	MH	-1H_20	R2
	953.41	+1	222	b5		R2
	892.48	+1	161	MH		R3
	875.63	+1	48	MH	-1NH_3	R3
	840.34	+1	130	MH	-1H_20/-2NH_3	R3
	718.45	+1	554	a5		R3
	527.42	+1	75	a4	-1NH_3	R3

C. Ion assignments for the MS2 spectrum of MH^{2+} 658.80 (KKQEEK) and MH^{2+} 773.37 (KKQEEKKTK) ions of the **1b** crosslinked peptides.

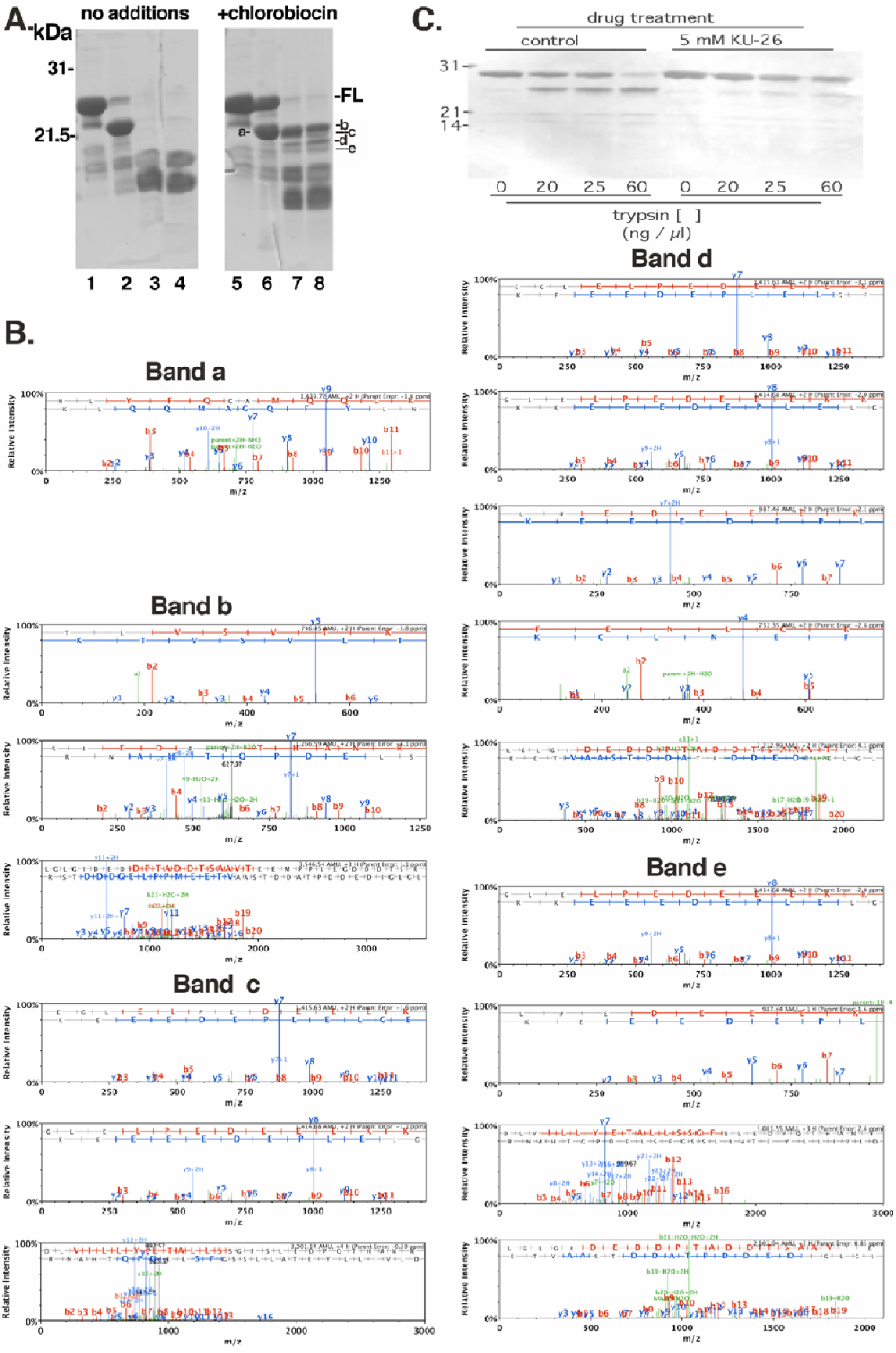
Parental Ion (m/z)	Fragment (m/z)	Charge	Intensity	Ladder Ion	Neutral Loss H_2O / NH_3	R
658.80		+2		MH		R1
	1100.52	+1	706	b5	-2H_20/-2NH_3	R1
	1099.48	+1	2203	MH		R2
	718.36	+1	14	a5		R3
	695.36	+1	10	b3		R2
	599.43	+1	49	b4	-1H_20	R3
	567.43	+1	20	b2		R2
	550.47	+2	1310	MH		R2
	499.36	+1	24	y4	-2NH_3	R3
	446.23	+2	9	MH		R3
	389.37	+1	18	z3		
	218.12	+1	330	Noviose		
773.37		+2		MH		R1
	1399.60	+1	194	b7		R1
	1381.60	+1	183	b7	-1H_20	R1
	1228.62	+1	194	b6	-2H_20/-2NH_3	R1
	1024.52	+1	355	b4	-1H_20	R1
	764.55	+2	18385	MH	-1H_20	R1
	756.53	+1	496	a2		R1
	614.59	+2	622	b6	-2H_20/-2NH_3	R1
	504.31	+1	94	y4		R1

Supplementary Table 2. Ion assignments of MS3 spectrum of ions present in the MS2 spectrum of the MH^{2+} 658.80 and MH^{1+} 1316.60 ions from peptides generated by **2** crosslinking to KQEEK.

MS2 Ion (m/z)	Fragment (m/z)	Charge	Intensity	Ladder Ion	Neutral Loss H_2O / NH_3	R
1099.38 from MH^{2+} 658.80				MH		R2
	1082.38	+1	429	MH	-1NH_3	R2
	1081.42	+1	153	MH	-1$H_2$0	R2
	971.36	+1	30	y5 or (b5 + $H_2$0)		R2
	953.40	+1	410	b5		R2
	925.40	+1	22	a5		R2
	892.55	+1	387	MH		R3
	875.52	+1	109	MH	-1NH_3	R3
	840.28	+1	332	MH	-1$H_2$0/-2NH_3	R3
	746.36	+1	34	b5		R3
	718.45	+1	554	a5		R3
	712.34	+1	78	b5	-2NH_3	R3
	701.44	+1	39	a5	-1NH_3	R3
	572.27	+1	210	a4	-1NH_3	R3
	516.38	+1	25	y4	-1NH_3	
	499.36	+1	37	y4	-2NH_3	
	388.27	+1	16	y3	-1NH_3	
1099.41 from MH^{1+} 1316.60				MH		R2
	1082.53	+1	105	MH	-1NH_3	R2
	1081.62	+1	59	MH	-1$H_2$0	R2
	971.37	+1	5	y5 or (b5 + $H_2$0)		R2
	953.31	+1	130	b5		R2
	925.40	+1	22	a5		R2
	892.55	+1	114	MH		R3
	875.63	+1	36	MH	-1NH_3	R3
	874.52	+1	6	MH	-1$H_2$0	R3
	840.34	+1	89	MH	-1$H_2$0/-2NH_3	R3
	764.52	+1	130	y5 or (b5 + $H_2$0)		R3
	718.45	+1	554	a5		R3
	712.29	+1	22	b5	-2NH_3	R3
	701.44	+1	19	a5	-1NH_3	R3
	572.39	+1	53	a4	-1NH_3	R3
	555.38	+1	10	a4	-2NH_3	R3
718.42 from MH^{1+} 1316.60				a5		R3

701.49	+1	25	a5	$-1NH_3$	R3
700.84	+1	554	a5	$-1H_20$	R3
683.42	+1	10	a5	$-1H_20/-1NH_3$	R3
572.27	+1	40	a4	$-1NH_3$	R3
555.32	+1	13	a4	$-2NH_3$	R3
554.30	+1	10	a4	$-1H_20/-1NH_3$	R3
341.10	+1	2	internal (y/QEE/a)	$-1H_20$	
239.91	+1	5	internal (y/QE/b)	$-1H_20$	
550.21 from MH^{2+} 658.80	+2		MH		R2
764.34	+1	55	y5		R3
718.48	+1	70	a5		R3
446.79	+2	96	MH		R3
382.23	+2	38	c5		R3
389.28 from MH^{2+} 658.80	+1		z3 (EEK)		
371.26	+1	43	z3 (EEK)	$-1H_20$	
354.32	+1	72	z3 (EEK)	$-1H_20/-1NH_3$	
353.27	+1	62	z3 (EEK)	$-2H_20$	
276.47	+1	68	y2		
258.21	+1	47	y2	$-1H_20$	
243.20	+1	72	EE (z/EEK/b)	–y1(K)	
215.18	+1	13	EE (z/EEK/a)	–(CO-K)	
147.02	+1	358	y1		
130.09	+1	128	y1	$-1NH_3$	
129.12	+1	85	y1	$-1H_20$	

SUPPLEMENTARY FIGURES


A.
kDa
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-FL
e-
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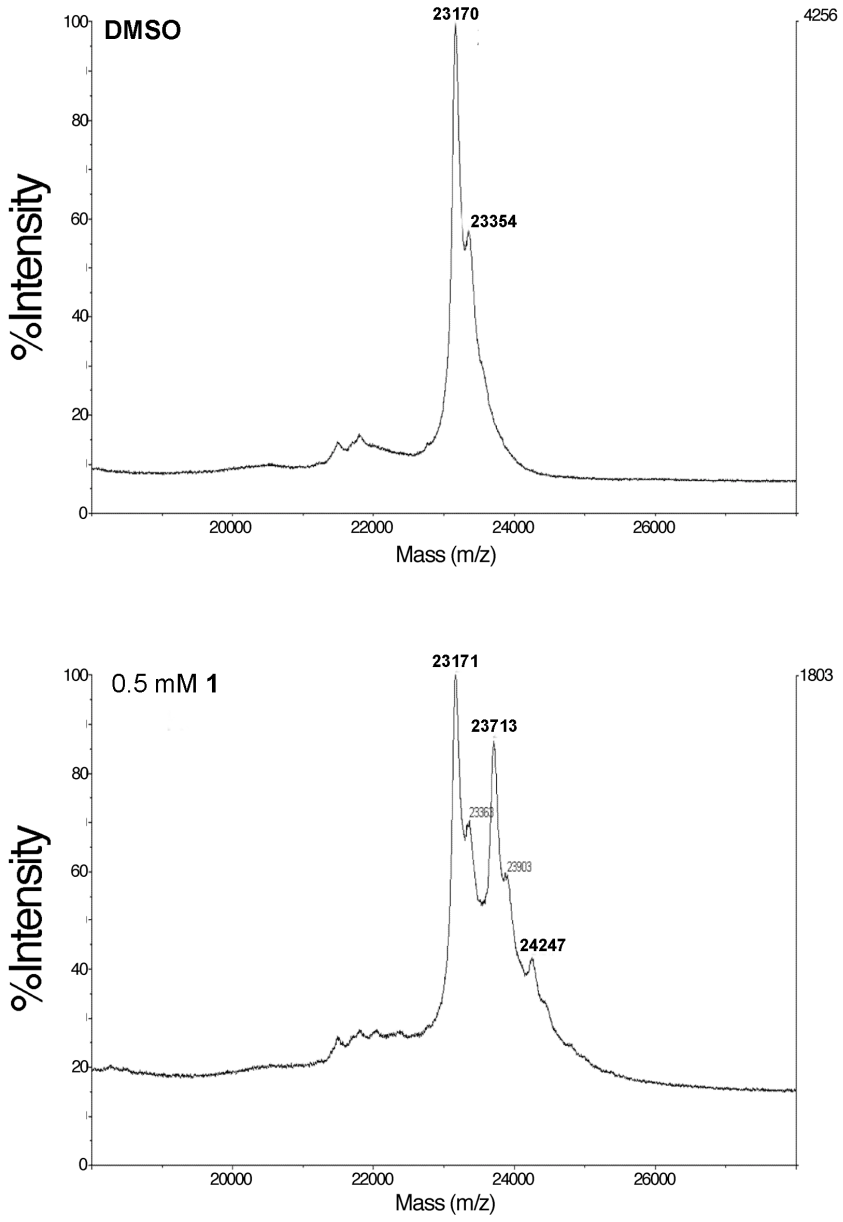
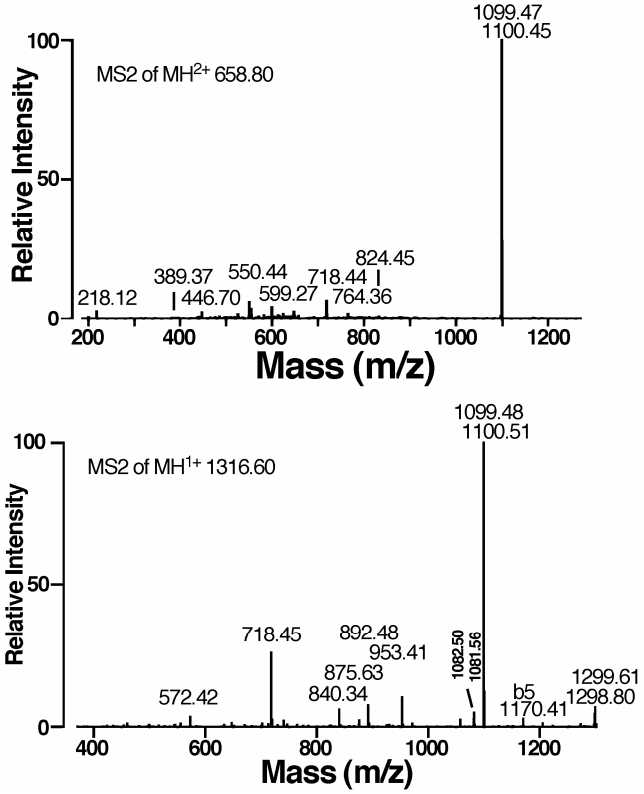
Band a
Band b
Band c
Band d
Band e
Relative Intensity
m/z
C.
drug treatment
control
5 mM KU-26
31-
21-
14-
0 20 25 60 0 20 25 60
trypsin [ ]
(ng / µl)

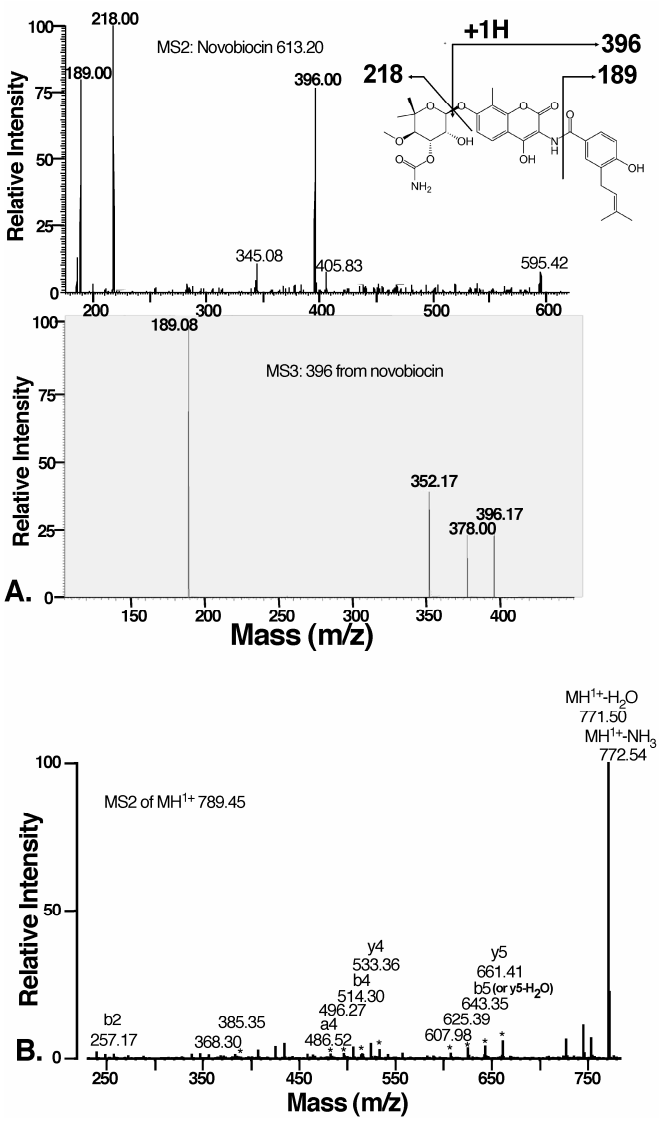
Supplementary Figure 1: Protease resistance of Hsp90CT induced by the binding of NB-derivatives. A. *Protease-resistance of chlorobiocin bound Hsp90CT*. Hsp90CT was incubated in the presence (lanes 2-4 & 6-8) of V8 protease with the addition of DMSO (vehicle control: lanes 1-4) or 800 mM Chlorobiocin (lanes 5-8) for 0 min (lanes 2 & 6), 15 min (lanes 3 & 7) or 30 min (lanes 4 & 8). Lanes 1 and 5: Undigested full length Hsp90CT (-FL). Proteases resistant bands a-e as indicated. **B.** *Representative MS2 spectra of peptides generated by trypsinolysis of protease-resistant bands a-e.* **C.** *Protease-resistance induced upon the binding of analog 1 binds to Hsp90-CT.* Hsp90-CT was incubated with 5 mM **1** (KU-26) or with drug vehicle (DMSO), for 30 minutes on ice. The reactions were then incubated for 6 minutes in presence of indicated concentrations of trypsin, analyzed by SDS-PAGE, and stained with Coomassie blue.



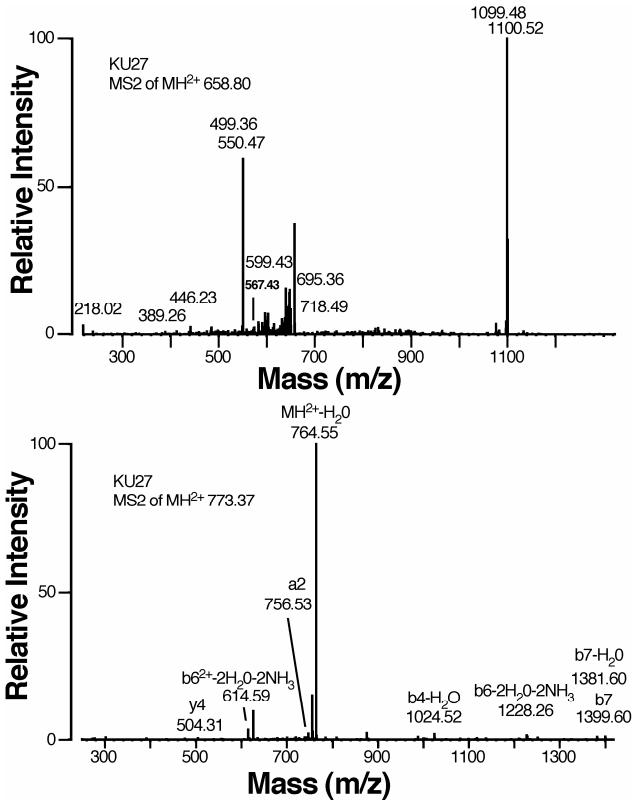
Supplementary Figure 2: Hsp90CT were UV irradiated as described under "Materials and Methods" in the presence of DMSO or 0.5 mM **2** and analyzed by MALDI-TOF mass spectrometry.



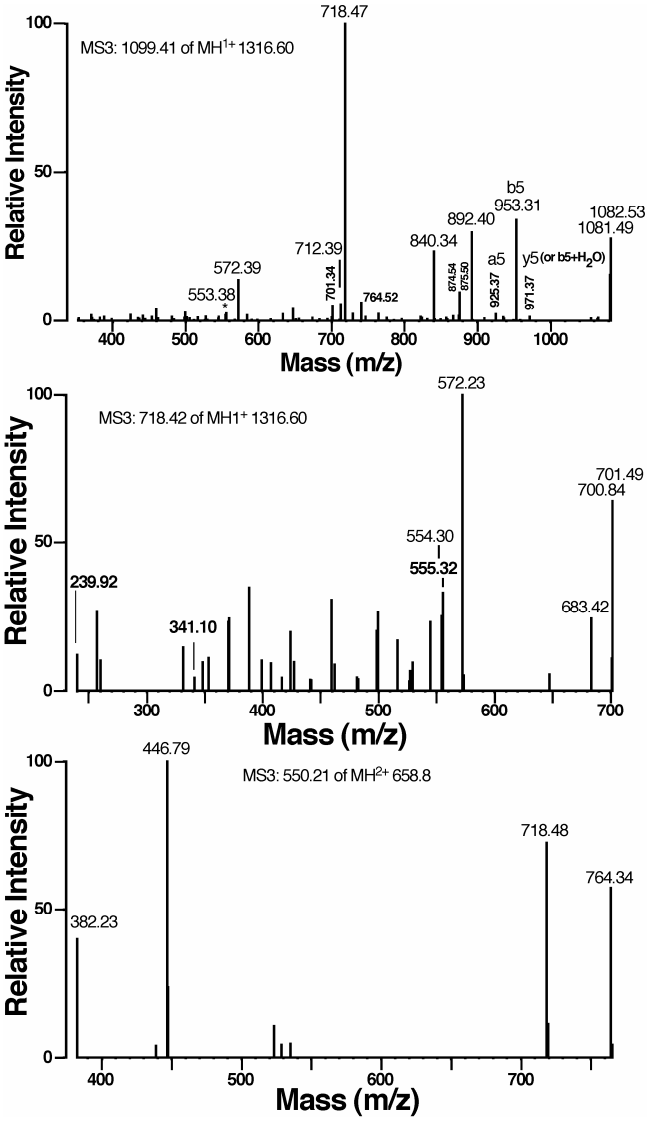
Supplementary Figure 3: MS2 spectrum of the major ions generated from crosslinking with compound 2. (**A**) MS2 spectrum of the MH^{2+} 658.80 ion and (**B**) MH^{1+} 1316.60 ion.



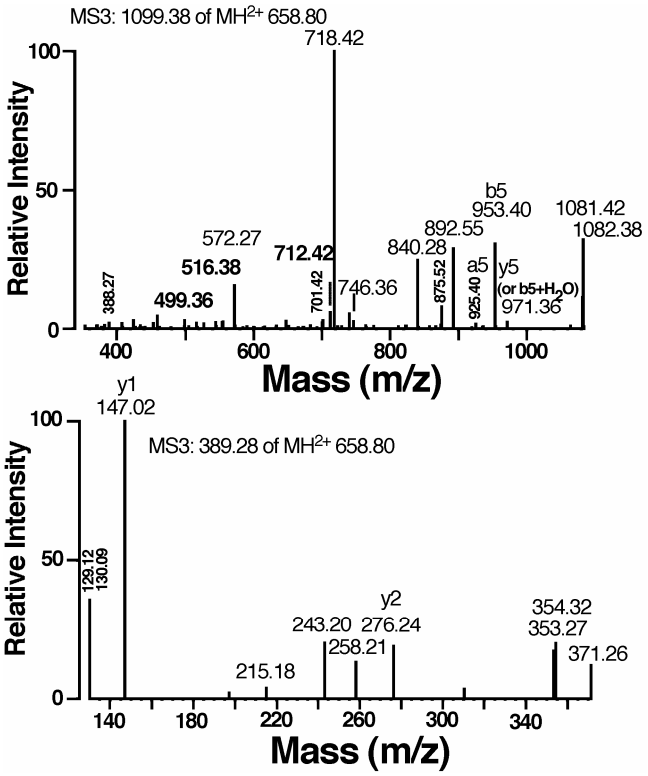
Supplementary Figure 4: MS spectrum used to aid in the interpretation of fragmentation patterns of the crosslinked peptide. A. MS2 spectrum of novobiocin and MS3 spectra of its 396 m/z fragment. **B.** MS2 spectrum of MH^{1+} ion 789.45.



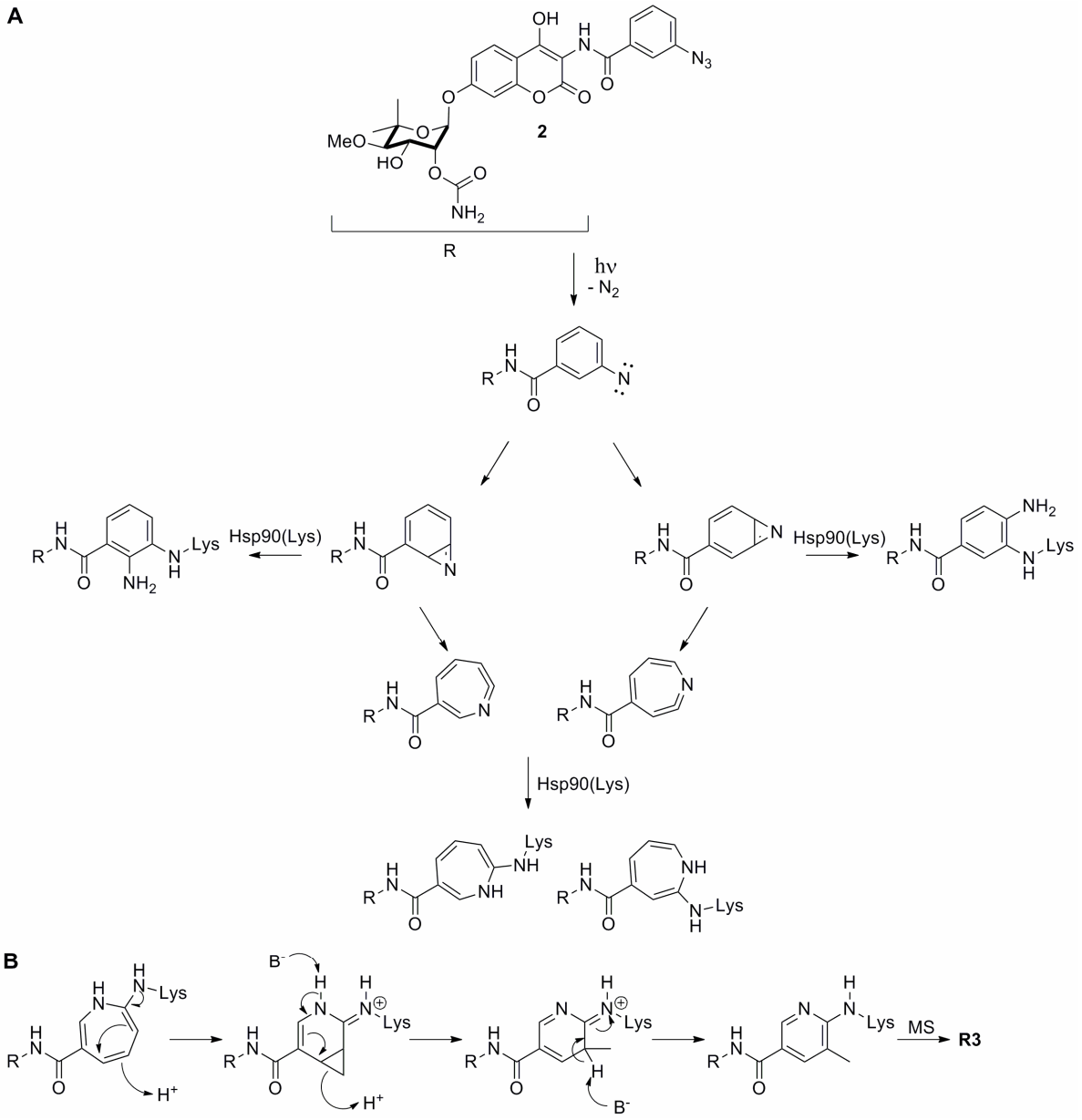
Supplementary Figure 5: MS2 spectrum of the MH^{2+} 658.8 and 773.37 ions from peptides generated by **1b** crosslinking.



Supplementary Figure 6: MS3 spectrum of the 1099.38 and 718.42 ions from MH^{1+} 1316.60 ion and 550.21 m/z ion from MH^{2+} 658.80 ion generated by **2** crosslinking.



Supplementary Figure 7: MS3 spectrum of ions generated from crosslinking with compound 2. (**A**) MS3 spectrum of the 1099.38 and (**B**) 389.28 m/z ion from MH^{2+} 658.80 ion.



Supplementary Scheme 1: A. Possible reaction products between peptide fragment and photo-label **2**. **B.** Rearrangement of the aminocycloheptatriene ring to give **R3**.

REFERENCES

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