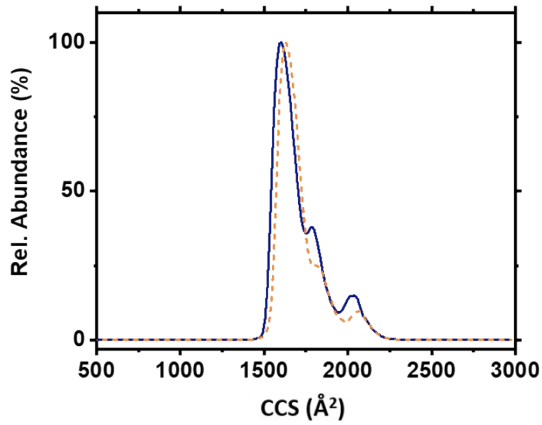
Electronic Supplementary Material (ESI) for ChemComm.

A New Native Mass Spectrometry Platform Identifies Inhibitors of the HSP90 – HOP Protein-Protein Interaction – Supplementary Information

A



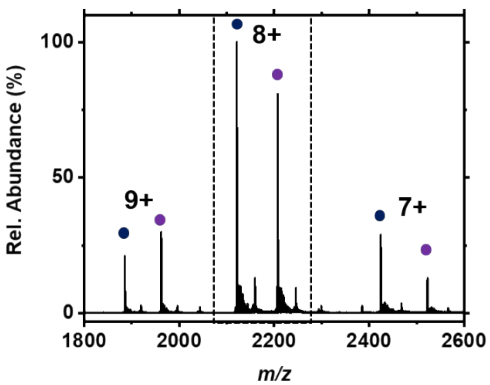
B

Theoretical CCS (Å²)	
TPR2A	TPR2A :1 complex
1655.75 ± 12.02	1662.1

Experimental CCS (Å²)	
TPR2A	TPR2A : 1 complex
1603.55 ± 161.1	1630.62 ± 151.7

Figure S1A: Ion mobility arrival time distribution of the 8+ charge state of TPR2A (blue solid line) and the TPR2A-AcMEEVD-OH complex (orange dashed line). **S2B.** CCS values of TPR2A and AcMEEVD-OH bound species determined theoretically (by analysis of 2NC9[#] and 1ELR) and experimentally from ion mobility analysis. The errors quoted for experimental values represent full width and half max (FWHM) values. [#]2NC9 is a solution structure, hence the margin of error in theoretical CCS

A



B

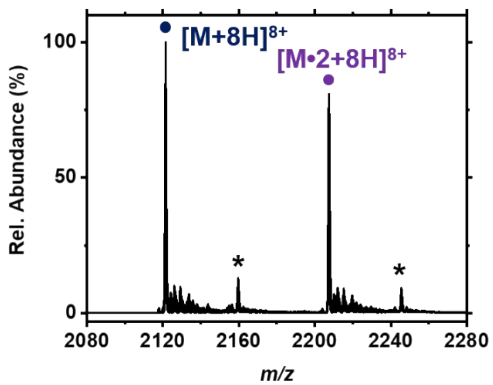


Figure S2A: Native MS spectrum of TPR2A and **2** (1:1) analysed by nESI from 50 mM ammonium acetate. **S2B:** Expanded view of the 8+ charge state. Blue, TPR2A; purple, TPR2A-AcMTrEVD; * protein impurities derived from protease cleavage during His-TPR2A purification.

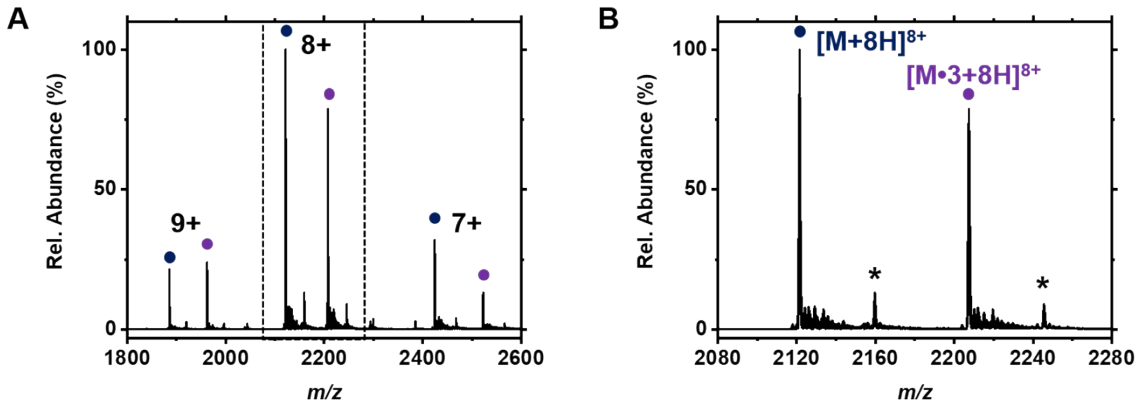


Fig S3A: Native MS spectrum of TPR2A and **3** (1:1) analysed by nESI from 50 mM ammonium acetate. **S3B:** Expanded view of the 8+ charge state. Blue, TPR2A; purple, TPR2A-AcMETrVD; *protein impurities derived from protease cleavage during His-TPR2A purification.

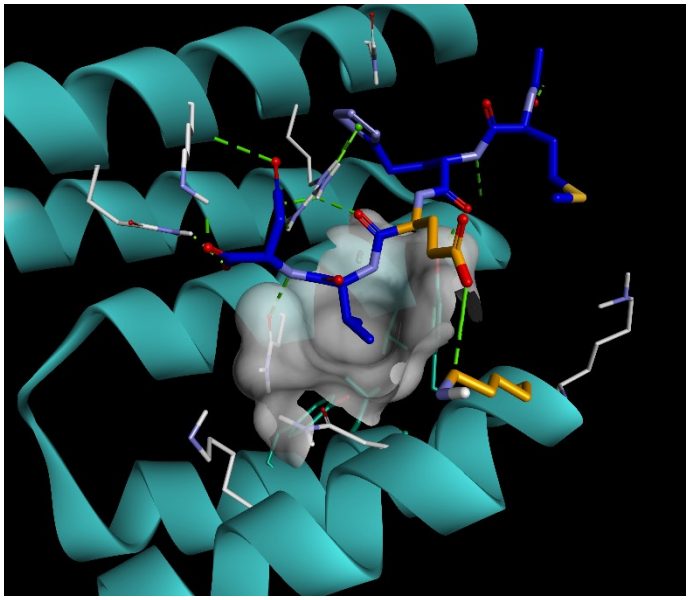


Figure S4: Lowest energy docked pose of peptide **2**

Experimental Details.

Mass Spectrometry

Protein expression and purification

His-tagged human TPR2A was expressed in E.coli and purified using Ni-charged IMAC column. Briefly, the TPR2A proEX-HTa plasmid was transformed into BL21(DE3) cells (New England Biolabs) and incubated in LB media containing ampicillin (100 µg/mL) at 37 ºC until the OD600 reading reached 0.6-0.7. Then, protein expression was induced by adding IPTG 1 mM (37 ºC) for 3 hours. Cultures were harvested (4000 ×g, 4ºC, 30 min) and stored frozen (-20 ºC). For protein purification, cell pellets were thawed and sonicated at 10 A (12 x 30 s bursts) on ice. The cellular debris was harvested (12000 ×g, 4ºC, 60 min). His-TPR2A was purified using a HisTrap HP column 1 mL (GE Healthcare Life Sciences) according to manufacturer's instructions. The His tag was cleaved with TEV protease at 4 ºC overnight and the soluble TPR2A was subsequently purified by affinity chromatography. The accurate mass of the protein was confirmed by LC-MS.

Sample Preparation for Native MS analysis

Protein samples were buffer exchanged into 50 mM NH_4OAc using Zeba Spin Desalting Column (Thermo Fisher Scientific) prior to MS analysis. The ligands were diluted in 50 mM NH_4OAc as required and preincubated with TPR2A (5 μM) in 96-well plates and held at 4 °C prior to MS analysis.

Native MS/IM-MS

Native MS and IM-MS data were obtained on both a Synapt-G2 Q-TOF (Waters) and a 12T SolariX 2XR FT-ICR (Bruker Daltonics). In both cases, ionisation was achieved using a NanoMate nESI infusion robot (TriVersa), sampling from a 96-well plate. Typically, a nanoelectrospay voltage of 1.55 kV was used and backing pressure was adjusted to maintain stable electrospray. For IM-MS analysis on the Synapt platform, typical Native MS conditions included a source temperature of 60 °C and a backing pressure of 5.26 mbar and nitrogen was used as the drift gas. MS data were processed using MassLynx v4.0 (Waters) and DriftScope v2.7 (Waters). Theoretical CCS values were calculated using IMPACT.[1]

For analysis on the FT-ICR platform a source temperature of 60 °C was used and the source and transfer optics were tuned to maintain noncovalent complexes. Typically, spectra were acquired as the sum of 200 1 MegaWord FID transients. DataAnalysis software (Bruker Daltonics) was used for the ratiometric analysis of different protein species. A mass list was generated using the FTMS algorithm (S/N threshold of 4). Then, the intensity of the most abundant isotopic peak from each of the three native charge states (9+, 8+ and 7+) was

combined for each protein species. The summed abundance of for each species was used to determine the relative ratio of each of the TPR2A and TPR2A-peptide bound species.

Table S1. Relative ratios of molecular ions resulting from binding of TPR2A to peptides 1, 2 and 3

TPR2A:MEEVD 1:1		TPR2A:MEEVD:MTrEVD 1:1:1			TPR2A:MEEVD:METrVD 1:1:1		
TPR2A	TPR2A-MEEVD	TPR2A	TPR2A-MEEVD	TPR2A-MTrEVD	TPR2A	TPR2A-MEEVD	TPR2A-MTrEVD
61.4 ± 1.69	38.6 ± 1.69	47.4 ± 4.42	20.8 ± 4.56	31.8 ± 0.265	47.8 ± 2.18	18.6 ± 1.18	33.6 ± 2.11

Experiments conducted in technical triplicate. Values are average ± SD

Peptide Synthesis

Peptides were synthesized manually at 0.1 mmol scale by Fmoc/tBu protocol in a syringe fitted with a porous polyethylene disc and attached to a vacuum trap for easy filtration. The syntheses were carried out on 2-CTC resin (loading 0.86 mmol/g) which was activated with 50% thionyl chloride in DCM for two hours. Then resin was washed properly with DCM and first coupling was done with Fmoc-Asp(tBu)-OH (3eq) in DCM in presence of N,N-diisopropylethylamine (DIEA) (10 eq) for two hours, then MeOH (50µL) was added for capping the unreacted Cl groups for 30 min at room temperature. For Fmoc removal treatment with 20% piperidine in DMF for 7 min was done at each cycle. The remaining couplings were done with a 3-fold excess of Fmoc-amino acid, N,N'-diisopropylcarbodiimide (DIC) and OxymaPure in 1:1:1 ratio for 45 min. At the end of the chain elongation, peptides were acetylated at the end terminus using acetic anhydride (10eq), DIEA (20eq) in DMF for 45 minutes prior to full deprotection and cleavage with TFA/H_2O/TIS (triisopropylsilane) (95:2.5:2.5 v/v, 90 min, RT). Peptides were precipitated by addition of chilled diethyl ether, taken up in water and lyophilized.

Peptide analysis.

Analytical HPLC was performed on an Agilent 1100 system using a Phenomenex Aeris^{TM}C18 (3.6µm, 4.6 × 150 mm) column, with flow rate of 1.0 mL/min and UV detection at 220 nm. Chemstation software was used for data processing. Solvent A was 0.1% (v/v) TFA in H_2O, solvent B was 0.1% (v/v) TFA in CH_3CN. Elution was done with linear gradient 5 to 95% of solvent B into A over 15 min. Preparative HPLC was performed on Phenomenex C18 (21.2 × 250 mm, 10 µm) LC-ESI-MS was performed on a Thermo Scientific Dionex UltiMate 3000 using Phenomenex Aeris^{TM}C18 (3.6 µm, 4.6 × 150 mm) column. Solvent A was 0.1% (v/v) formic acid in H_2O, solvent B was 0.1% (v/v) formic acid in CH_3CN.

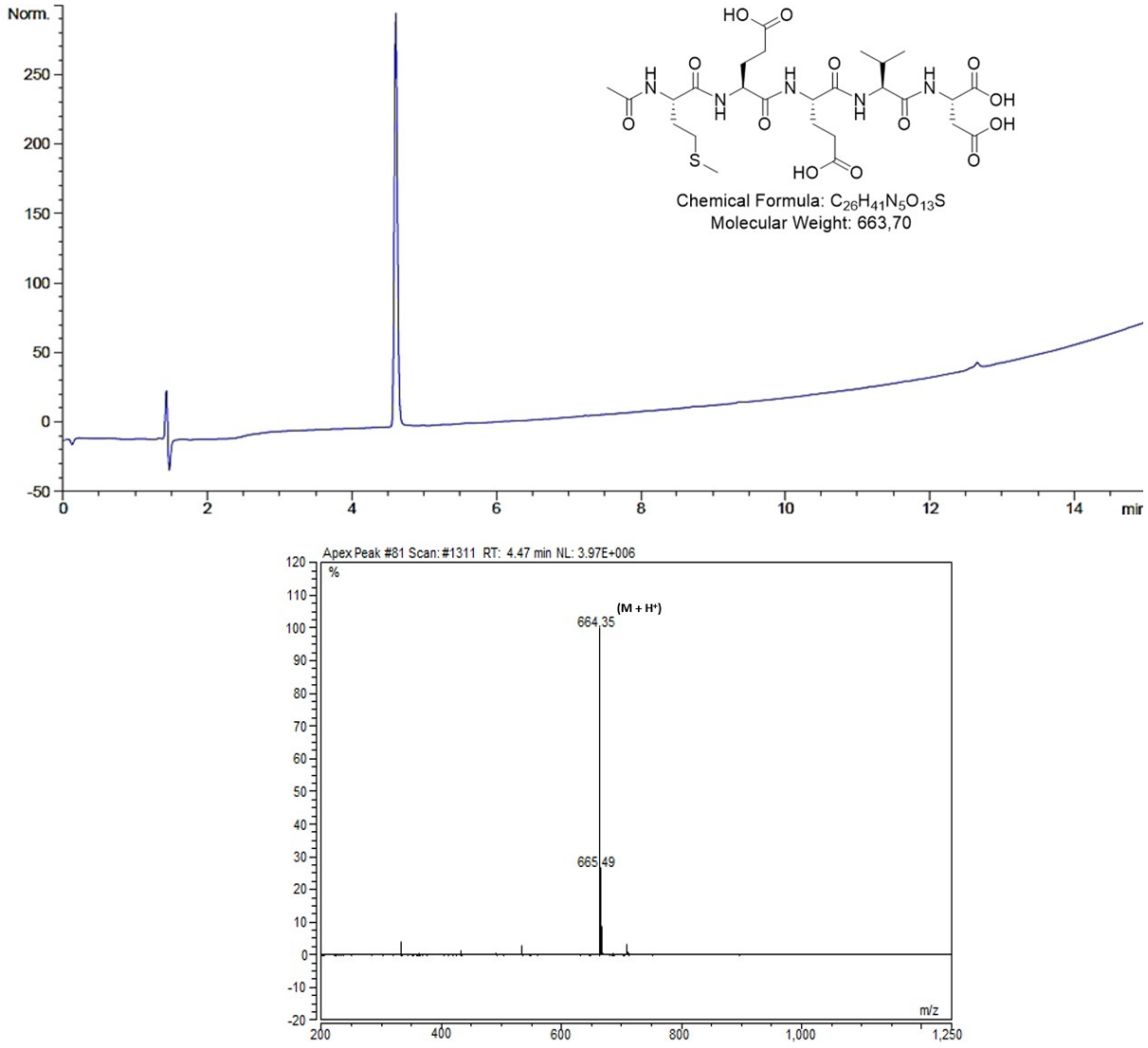


Fig S5 Top: Analytical HPLC trace of peptide **1**, using a solvent gradient 5 – 95%. Compound purity determined to be >99%. **Bottom**: ESI Mass spectrum of peptide **1** corresponding with predicted mass of peptide

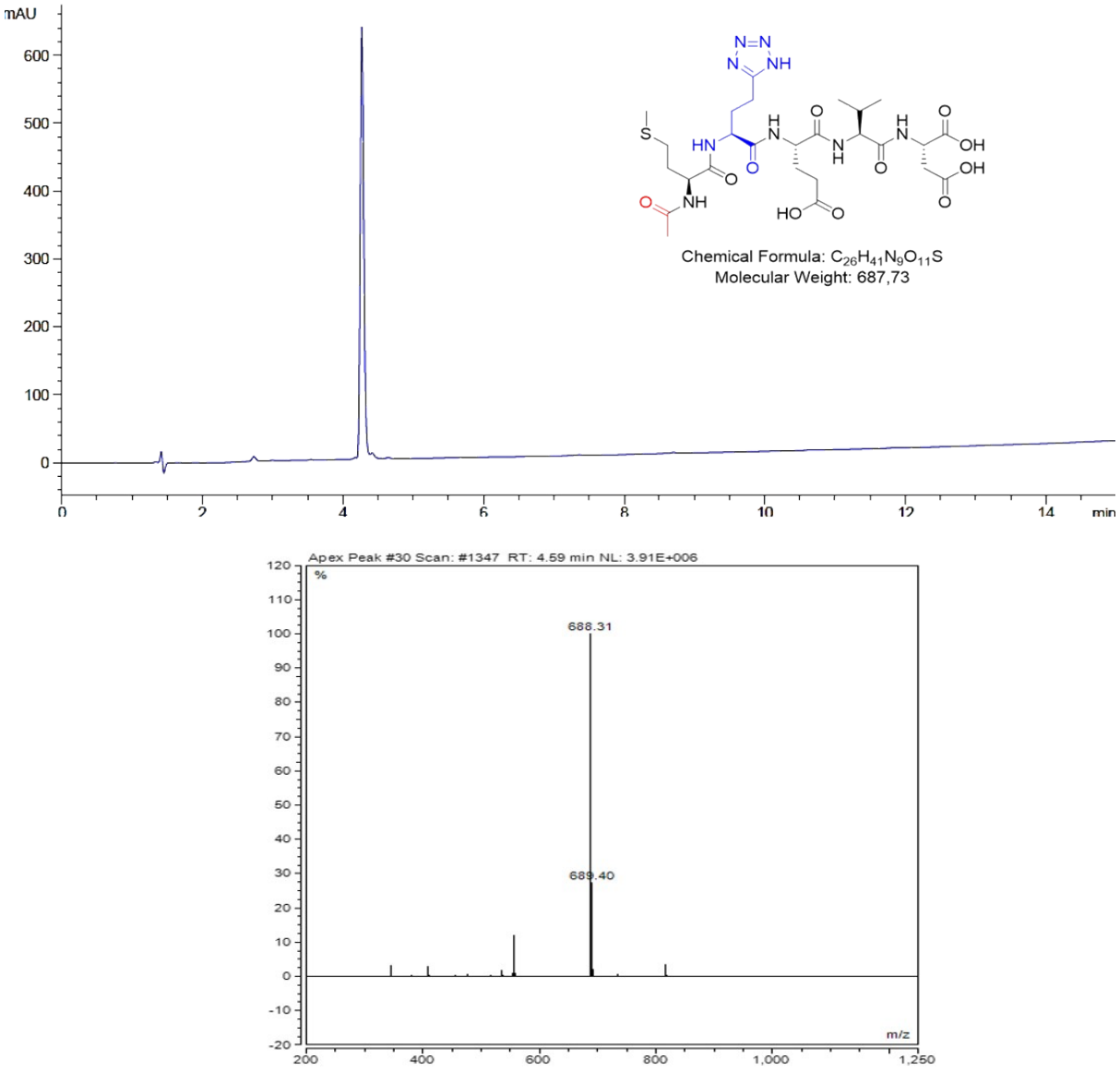


Fig S6 Top: Analytical HPLC trace of peptide **2**, using a solvent gradient 10 – 60%. Compound purity determined to be >98%. **Bottom**: ESI Mass spectrum of peptide **2** corresponding with predicted mass of peptide.

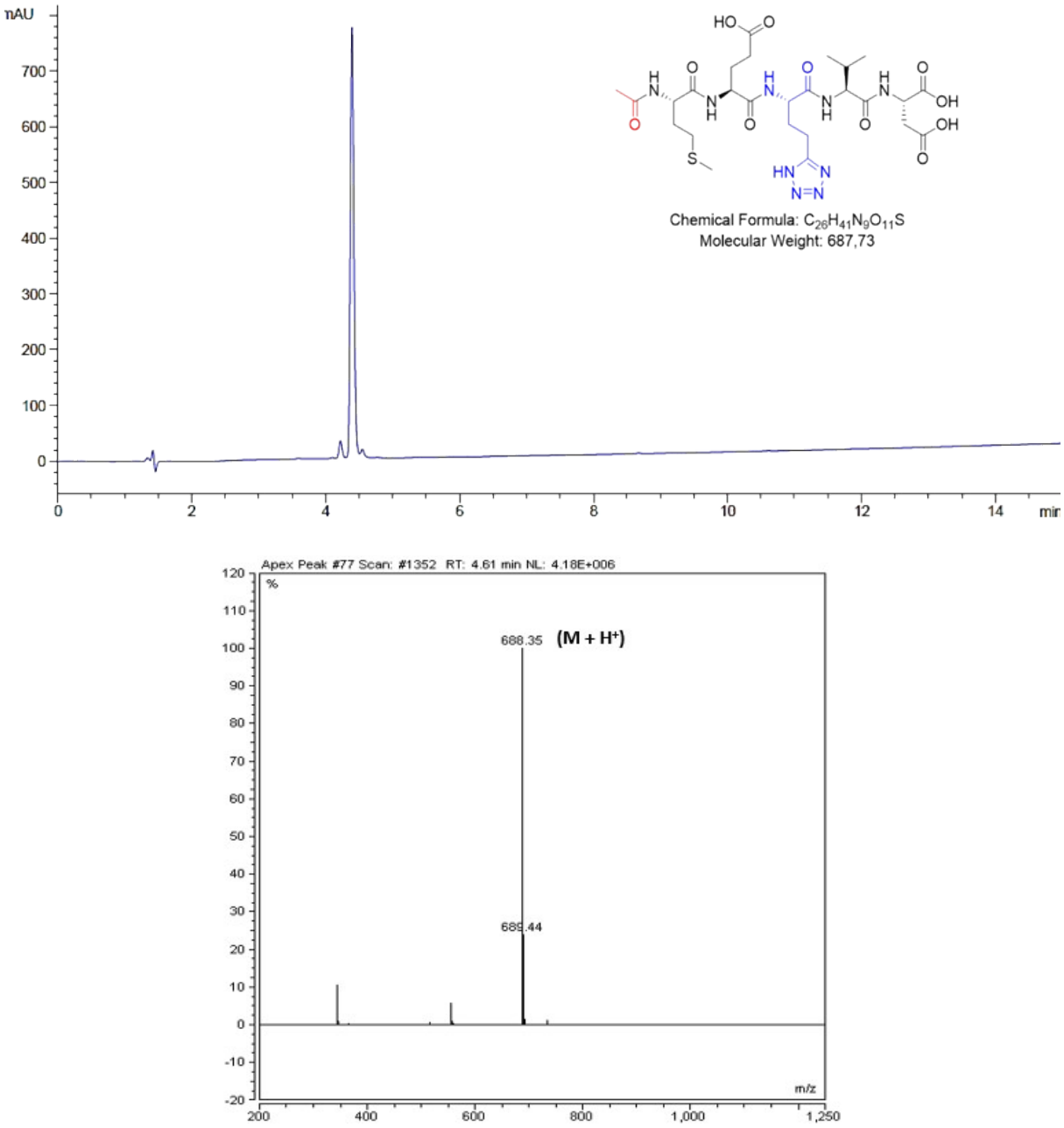
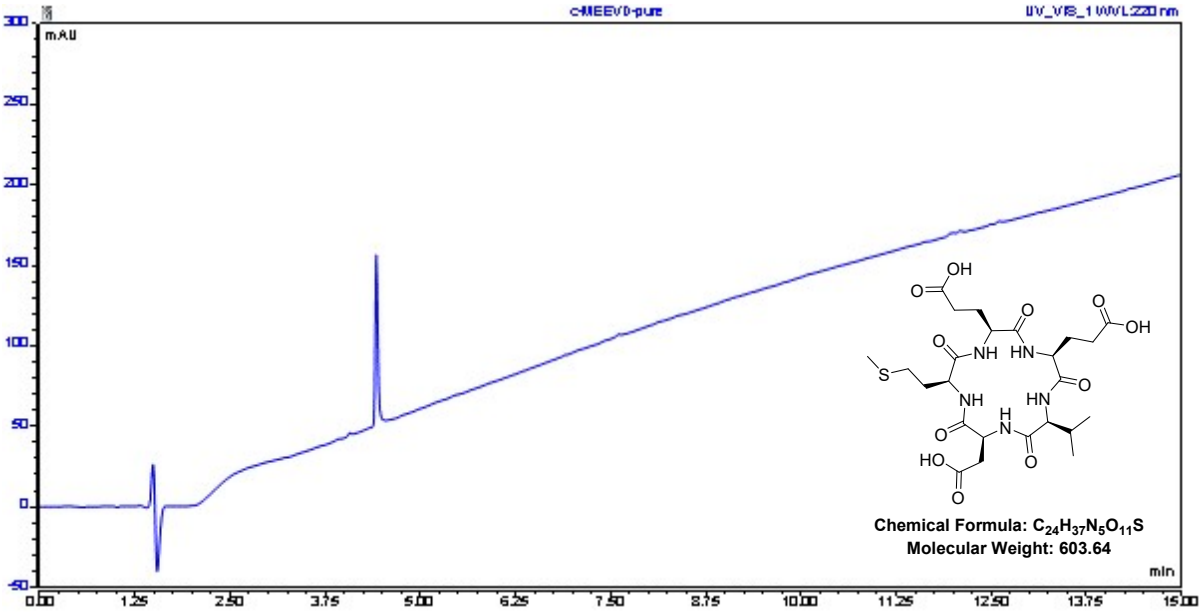


Fig S7 Top: Analytical HPLC trace of peptide **3**, using a solvent gradient 10 – 60%. Compound purity determined to be >95%. **Bottom**: ESI Mass spectrum of peptide **3** corresponding with predicted mass of peptide.



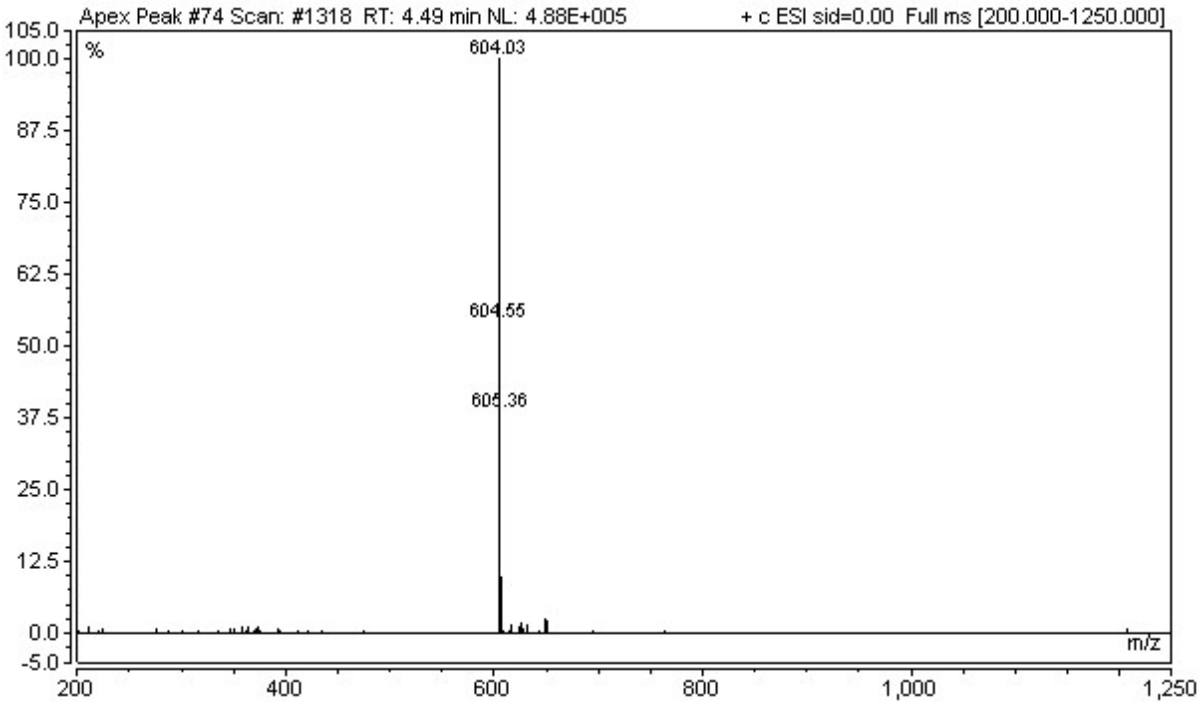


Fig S8 Top: Analytical HPLC trace of peptide **4**, using a solvent gradient 10 – 60%. Compound purity determined to be >99%. **Bottom**: ESI Mass spectrum of peptide **4** corresponding with predicted mass of peptide.

Biological assessment

Recombinant protein production and purification

Recombinant proteins were produced in E.coli. GST was produced from the pGEX-4T-1 plasmid, while GST-Hsp90αC (residues 626-732 of Hsp90α) was produced from the pGEX-4T3-Hsp90α-C (626-732) plasmid which was a gift from William Sessa (Addgene plasmid #22483; http://n2t.net/addgene:22483; RRID:Addgene_22483).[2] His-mSTI1 was produced from the pQE30-2000 plasmid.[3] The His-SUMO-TPR2A protein was produced from the pCA258-SUMO-TPR2A plasmid (encompassing residues 201 to 360 of human Hop). The pCA258 backbone was a gift of Matthias Mayer (ZMBH, Heidelberg, Germany). The murine (mSTI1) and human (Hop) versions of the protein share 98 % amino acid identity.[4] GST-tagged proteins and His-tagged proteins were purified from bacterial lysates by glutathione affinity chromatography or nickel-nitrilotriacetic acid affinity chromatography, respectively, according to established protocols.[5]

Solid phase binding analysis for protein- protein interactions

Interaction between GST-Hsp90C and either His-mSTI1 or His-SUMO-TPR2A was assessed as previously described.[6] His-mSTI1 (100 µg/mL) or His-SUMO-TPR2A (100 µg/mL) were coated on a high-binding ELISA plate in buffer A [50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05 % (v/v) Tween]) for 30 min at room temperature, followed by overnight at 4 °C. Non-specific binding sites were blocked with 1 hour room temperature incubation with 3% (w/v) or 5 % (w/v) BSA in Buffer A, for plates with His-SUMO-TPR2A and His-mSTI1, respectively. For the His-mSTI1 plate, GST-Hsp90αC (1 µM) in Buffer B (Buffer A+ 0.1 % [w/v] BSA) were incubated alone or with treatments (peptides or novobiocin) overnight at 4 °C. For the His-SUMO-TPR2A plate, the peptides were incubated for 30 minutes at room temperature and 16 hours overnight at 4 °C, after which GST-Hsp90αC (1 µM) was added and incubated for 2 hours at room temperature. Thereafter, both sets of plates were washed thrice with 1% (w/v) BSA in buffer A . The His-mSTI1 plate was incubated with rabbit anti-GST antibody (1 in 5000) in buffer A for 2 hrs at room temperature. The His-SUMO-TPR2A plate was incubated with mouse anti-GST primary antibody (1 in 1000 dilution) in buffer A for 16 hours at 25 °C.. Primary antibody incubation was followed by three washes in 1 % (w/v) BSA and incubation with species-matched secondary antibody (1 in 5000 dilution) for 2 hours at room temperature. After three washes with 1 % (w/v) BSA, HRP substrate [0.05 M phosphate-citrate buffer, 0.005 % (v/v) H_2O_2 , 1 mg/mL 3,3′,5,5′-Tetramethylbenzidine, 1% v/v DMSO] was added and incubated in the dark for at least 20 minutes at room temperature. The reaction was stopped with addition of 2M H_2SO_4 and the absorbance read at 450 nm.

In silico assessment

Molecular mechanics simulations were performed using the Schrödinger Maestro software suite.[7] The protein structure of interest, 1ELR,[8] was downloaded from the Protein Data Bank. The protein was optimised at pH 7,0 and bad contacts were removed using the protein preparation wizard. The protein was thereafter subjected to QM-MM optimisation using the QSite module and the DFT-B3LYP method together with the OPLC_2005 force field. Distance dependant dielectrics were applied. Finally, the docking centroid were selected to include an area of 10 Å from the MEEVD peptide. The peptides were sketched at pH 7,0 and tautomers were generated using the LigPrep module. Conformations were generated using the ConfGen module. Peptides were docked using the Glide module with the OPLS3e force field and partial charges were applied. Flexible ligand sampling, a soften potential scaling factor of 0,8 and a partial charge cut-off of 0,15 were used. Finally, the docked ligands were subjected to a binding energy calculation using the Prime MM-GBA module, the OPLS3e force field and VGBD solvation model. The Glide Score, and MMGBSA dG Bind (binding energy in $kcal.mol^{-1}$) is reported.

(1) Marklund, E. G.; Degiacomi, M. T.; Robinson, C. V.; Baldwin, A. J.; Benesch, J. L. P. Structure **2015**, 23, 791–799.

(2) Fontana, J.; Fulton, D.; Chen, Y.; Fairchild, T. A.; McCabe, T. J.; Fujita, N.; Tsuruo, T.; Sessa, W. C. Circ. Res. **2002**, 90, 866–873.

(3) Van Der Spuy, J.; Kana, B. D.; Dirr, H. W.; Blatch, G. L. Biochem. J. **2000**, 345, 645–651.

(4) Blatch, G. L.; Lässle, M.; Zetter, B. R.; Kundra, V. Gene **1997**, 194, 277–282.

(5) Edkins, A. L.; Ludewig, M. H.; Blatch, G. L. Int. J. Biochem. Cell Biol. **2004**, 36, 1585–1598.

(6) Hunter, M. C.; O'Hagan, K. L.; Kenyon, A.; Dhanani, K. C. H.; Prinsloo, E.; Edkins, A. L. PLoS One **2014**, 9, e86842.

(7) Schrödinger Release 2019-2; Maestro, Schrödinger, LLC: New York, NY, 2019.

(8) Scheufler, C.; Brinker, A.; Bourenkov, G.; Pegoraro, S.; Moroder, L.; Bartunik, H.; Hartl, F. U.; Moarefi, I. Cell **2000**, 101, 199–210.