

Discovery and characterization of a cryptic secondary binding site in the molecular chaperone HSP70

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Supplementary Materials

Figure S1: HSP70 ATP binding site structures.

Figure S2: ATP interactions with HSP70.

Figure S3: ATP site structure in S275W HSC70.

Figure S4: Binding of **3** to HSP72-NBD.

Figure S5: Protein structural changes during ligand pulling MD simulation.

Figure S6: Validation of HSP70 docking protocol.

Figure S7: SPR profiles of vHTS hits binding HSC70-NBD.

Figure S8: The docked pose of compound **4** in the cryptic pocket of HSP70 as predicted by the ASP scoring function.

Figure S9: The docked pose of compound **5** in the cryptic pocket of HSP70 as predicted by the ASP scoring function.

Figure S10: The docked pose of compound **6** in the cryptic pocket of HSP70 as predicted by the ASP scoring function.

Figure S11: The docked pose of compound **7** in the cryptic pocket of HSP70 as predicted by the ASP scoring function.

Figure S12: The docked pose of compound **8** in the cryptic pocket of HSP70 as predicted by the ASP scoring function.

Figure S13: ¹H and ¹³C NMR spectra for compounds **1**, **2** and **3**.

Figure S14: LC-MS spectra for compounds **1** – **8**.

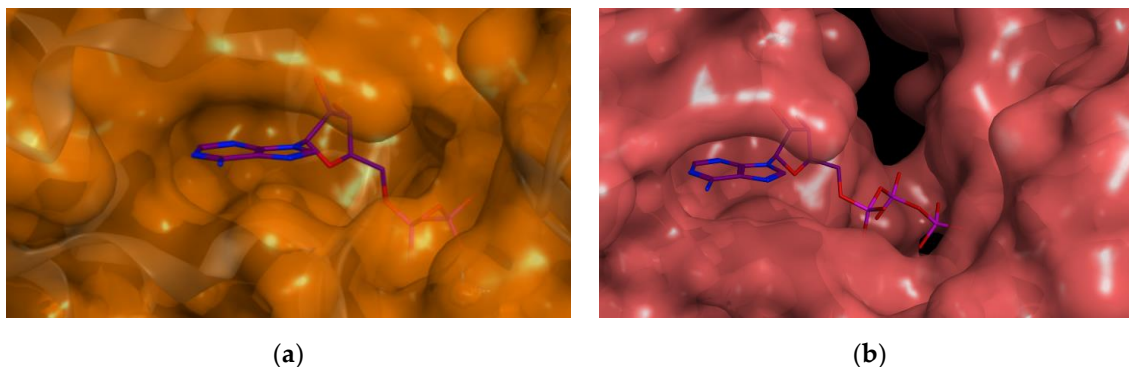
Figure S15: Sequence alignment of the nucleotide binding domains of human HSP72 and HSC70 proteins.

Figure S16: Determination of buffer solubility of **1** by quantitative NMR.

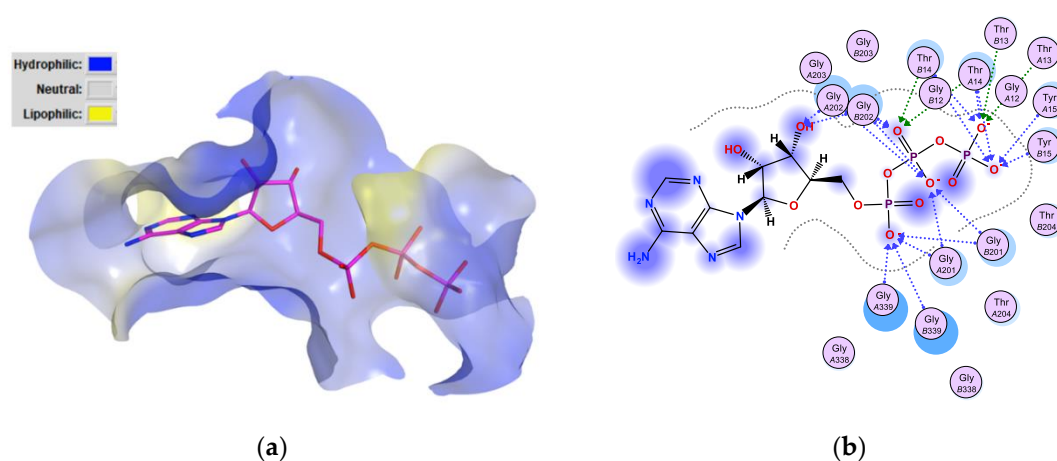
Table S1: Observed changes in NMR signal intensities for **3** on addition of HSP72-NBD and ATP.

Table S2: LC-MS data to confirm the identity and purity of vHTS hits **4** – **8**.

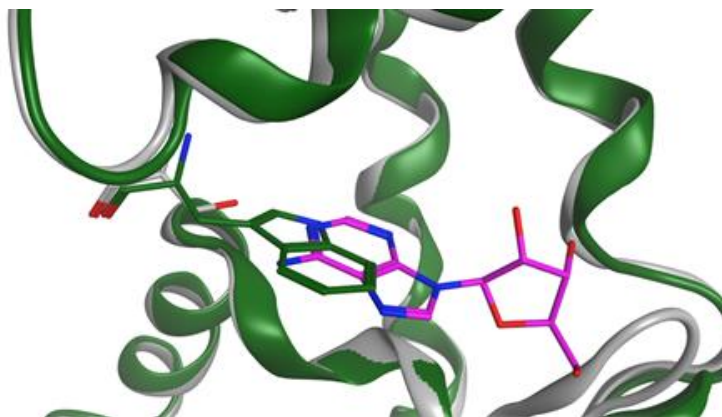
Table S3: Crystallographic data collection and refinement statistics for **1**-HSP72-NBD.



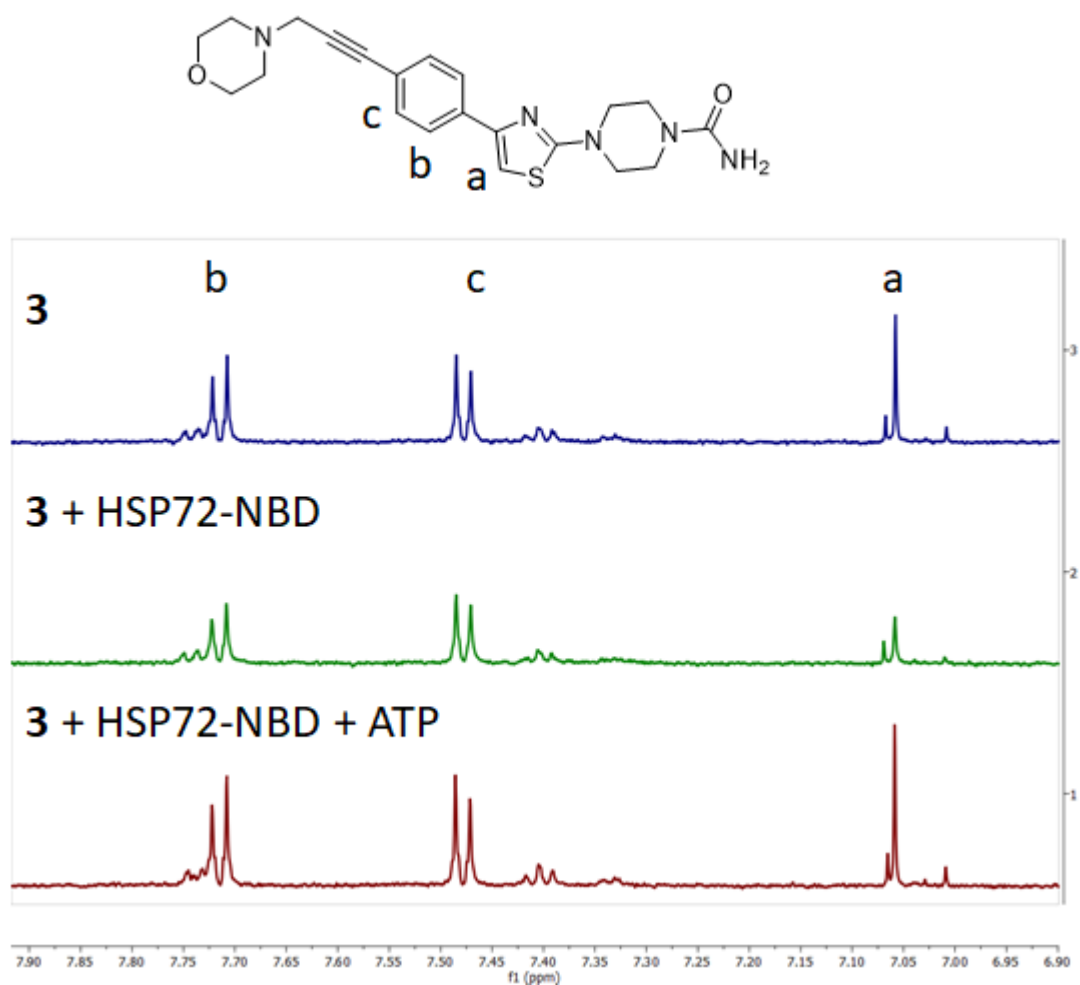
Supplementary Figure S1. HSP70 ATP binding site structures. The HSP70 ATP-binding site is highly flexible with the size and shape dependent on nucleotide binding and association with nucleotide exchange factors such as Bag1. **(a)** Structure of HSC70-NBD in complex with ADP (PDB ID: 4H5T). **(b)** Structure of HSC70/Bag1 in complex with ATP (PDB ID: 2KHO).



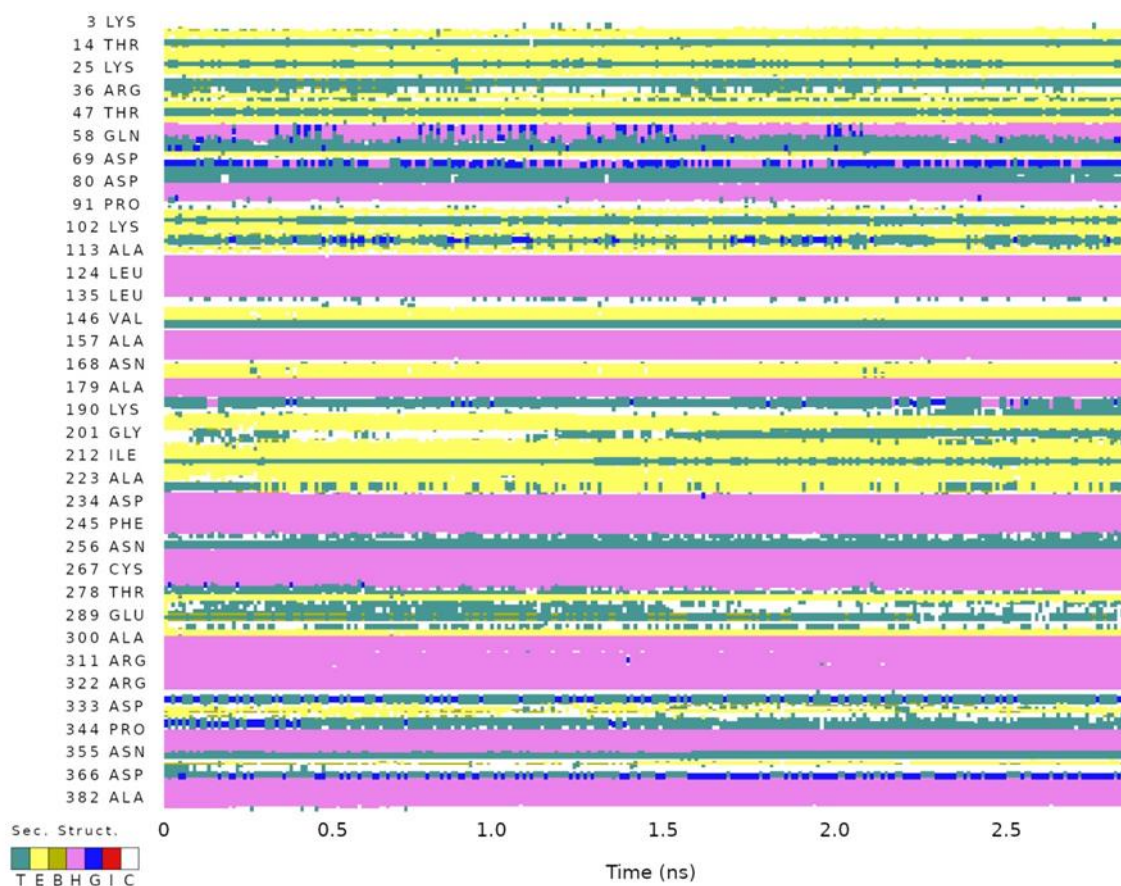
Supplementary Figure S2. ATP interactions with HSC70. **(a)** Representation of the surface of the ATP-binding site of HSC70/Bag 1 in complex with ATP (sticks; PDB ID: 3FZF) showing the prevalence of hydrophilic (blue) in comparison to lipophilic (yellow) and neutral (grey) residues on the surface. **(b)** Ligand interaction diagram showing the binding of the β - and γ -phosphates of ATP stabilized by interactions with polar amino acids, water and cations in the binding site.



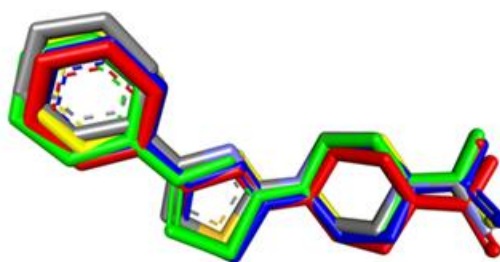
Supplementary Figure S3. ATP site structure in S275W HSC70. Overlay of wild type HSC70-NBD bound to adenosine (PDB ID 5AQF; grey) with the S275W mutant HSC70-NBD (PDB ID 5AQL; green). The large indole group of the tryptophan (green sticks) occupies the space where the adenine ring of adenosine (magenta sticks) would normally bind.



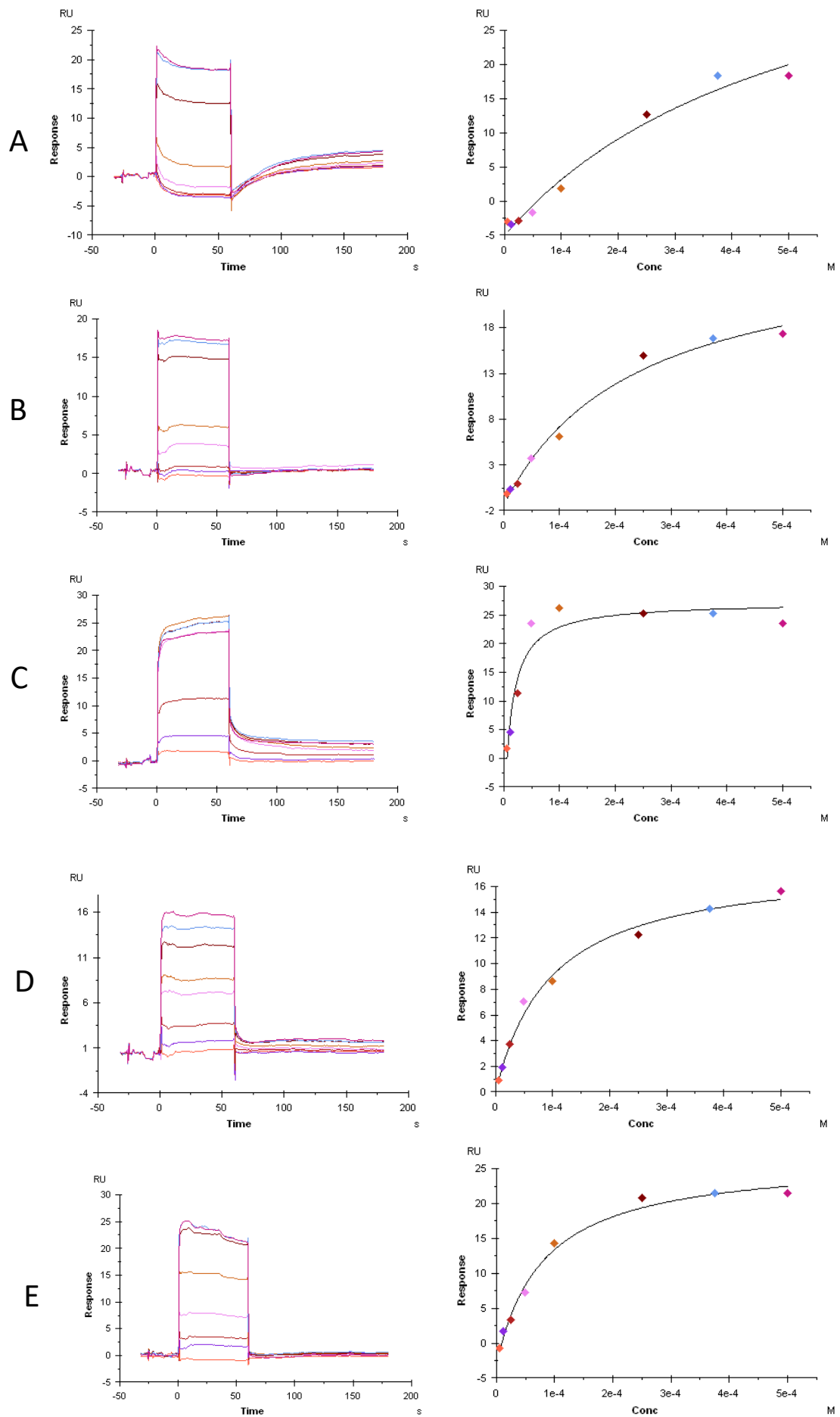
Supplementary Figure S4. Binding of 3 to HSP72-NBD was detected by CPMG LO-NMR and displaced by the addition of ATP.



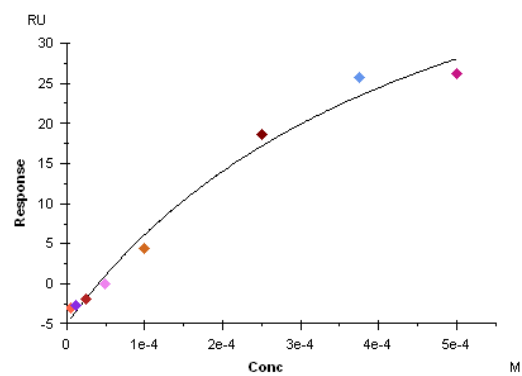
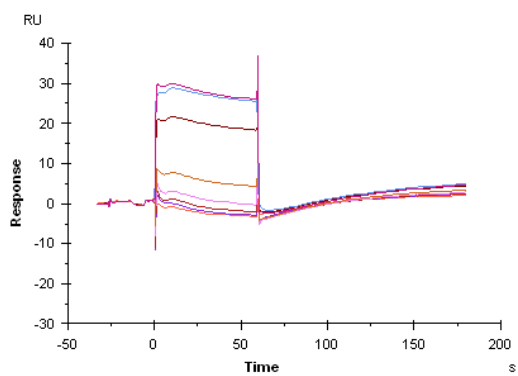
Supplementary Figure S5. Protein structural changes during ligand pulling MD simulation. Heat map representation of HSP72-NBD secondary structures showing they are broadly maintained during the MD pulling experiment to remove **1** from the secondary binding site; where T represents the turns, E the β -sheet, B the isolated bridge, H the α -helix, G the 3-10 helix, I the Π -helix and C the coil structures.



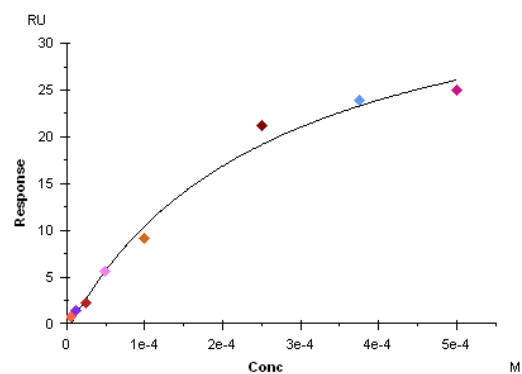
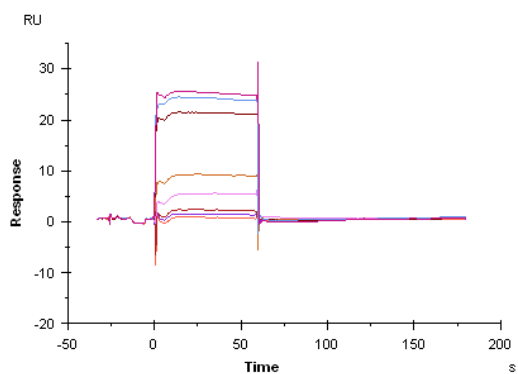
Supplementary Figure S6. Validation of HSP70 docking protocol. Overlays of the docked binding poses of **1** with the crystallographic data for **1**-HSP72-NB (PDB ID: 7Q4R). The crystal structure conformation of **1** is colored by element, the docked conformations are colored as: ChemPLP, blue; CS, red; GS, yellow, and ASP, green. The root-mean-square deviation (RMSD) between the heavy atoms of **1** in the crystal structure conformation and the predicted binding poses are: ChemPLP – 0.8422 Å, CS – 0.9190 Å, GS – 0.9602 Å and ASP – 0.7938 Å. RMSD values < 1 indicate excellent reproduction of the experimental situation.



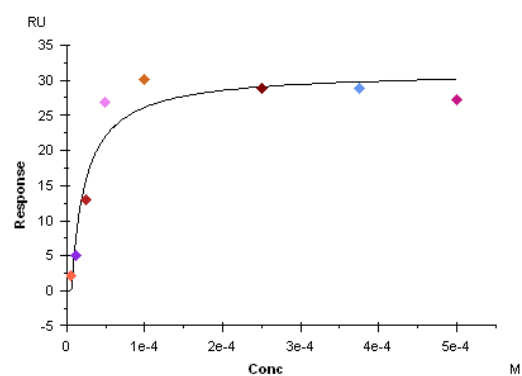
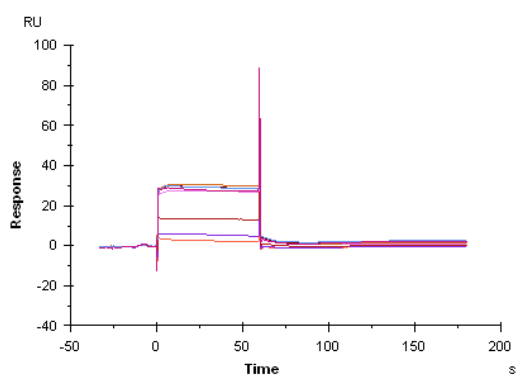
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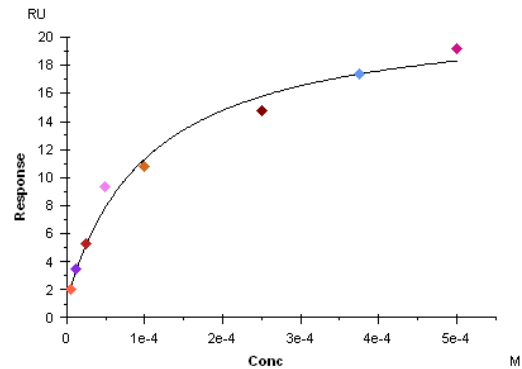
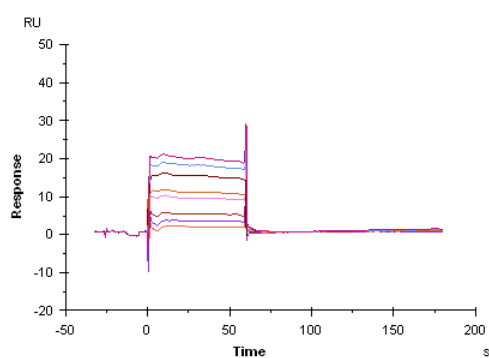
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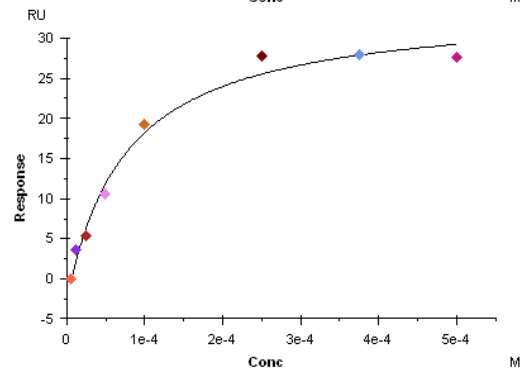
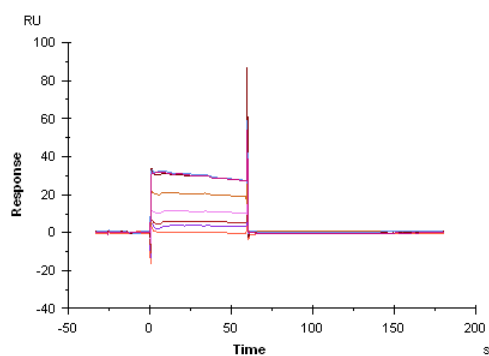
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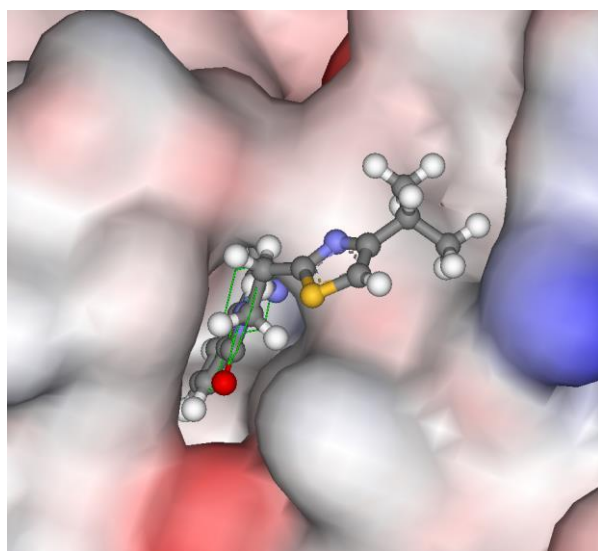
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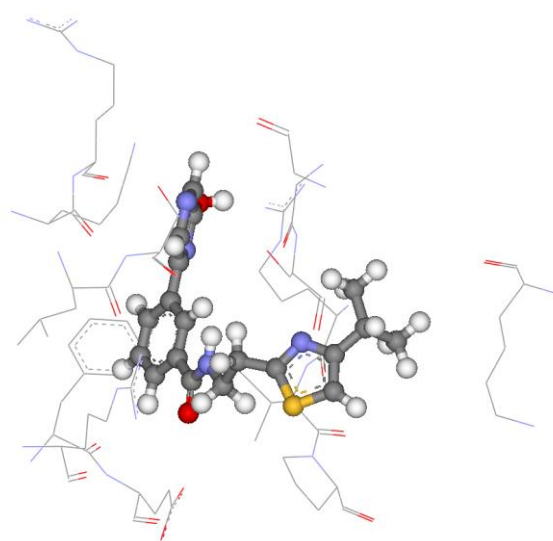
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Supplementary Figure S7. SPR profiles of vHTS hits binding HSC70-NBD. Representative SPR sensorgrams and Langmuir isotherms for vHTS hits (a) 4, (b) 5, (c) 6, (d) 7 and (e) 8 binding to WT HSC70-NBD. Representative SPR sensorgrams and Langmuir isotherms for vHTS hits (f) 4, (g) 5, (h) 6, (i) 7 and (j) 8 binding to S275W HSC70-NBD.

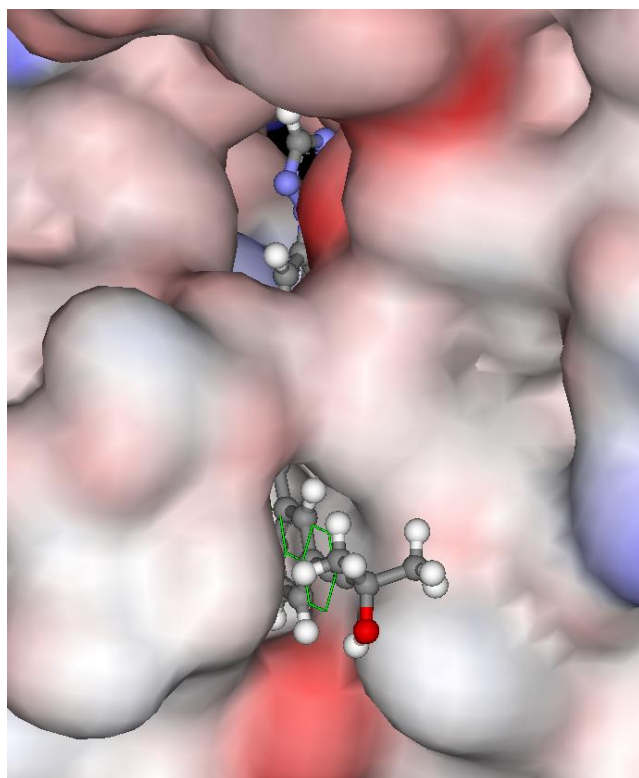


(a)

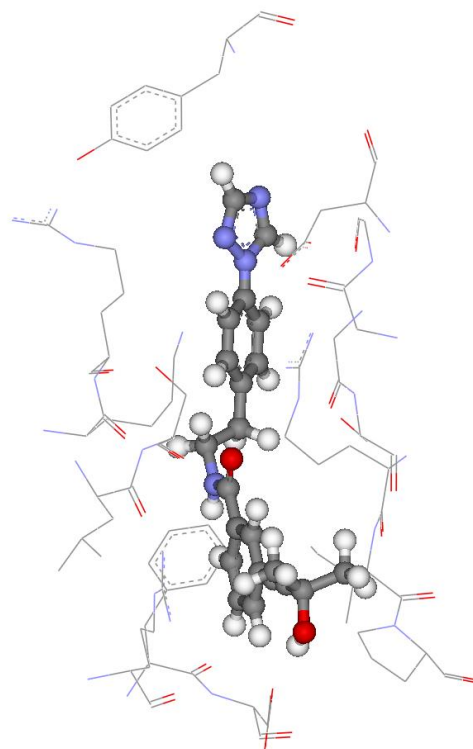


(b)

Supplementary Figure S8. The docked pose of compound **4** in the cryptic pocket of HSP70 as predicted by the ASP scoring function. **(a)** Compound **4** shown as ball-and-stick and the crystallized ligand **1** depicted as green lines. The protein surface shown where blue depicts regions with a partial positive charge on the surface, red depicts regions with a partial negative charge and grey shows neutral areas. **(b)** The predicted conformation depicted as ball-and-stick; no specific interactions are predicted between the ligand and the protein. The adjacent amino acids ($< 5 \text{ \AA}$), buttressing the ligand, are shown as lines. Hydrogen atoms of the amino acids are omitted for clarity.

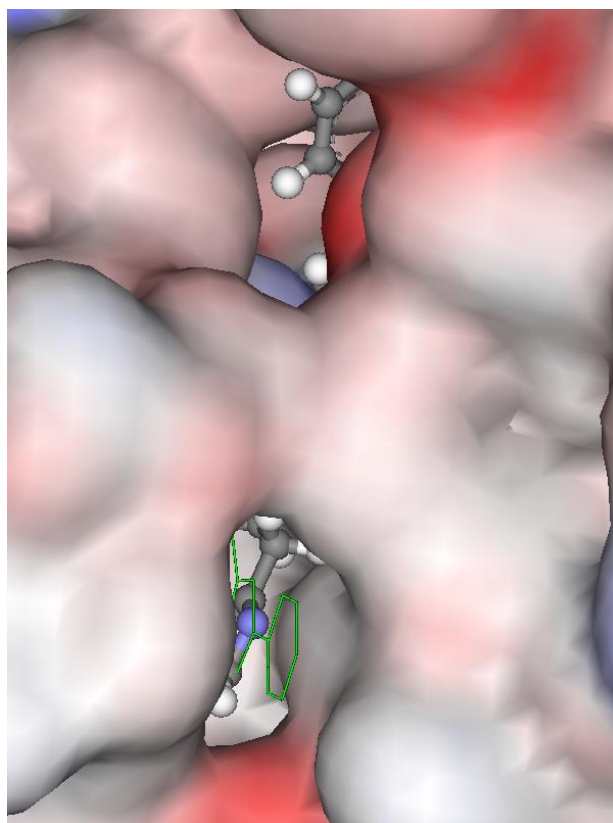


(a)

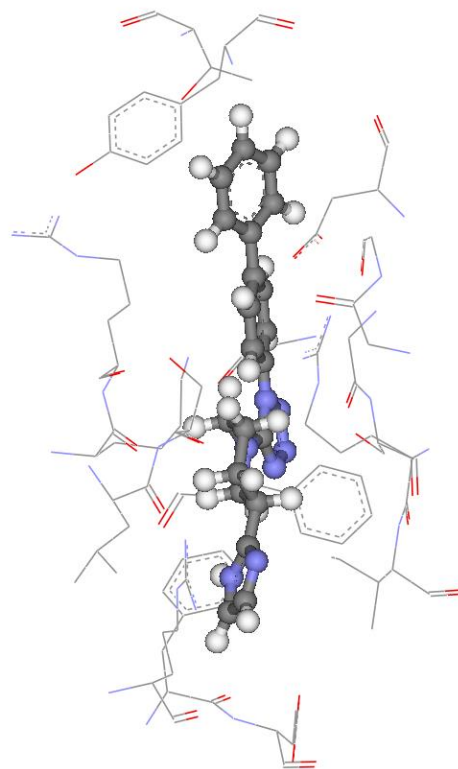


(b)

Supplementary Figure S9. The docked pose of compound **5** in the cryptic pocket of HSP70 as predicted by the ASP scoring function. **(a)** Compound **5** shown as ball-and-stick and the crystallized ligand **1** depicted as green lines. The protein surface shown where blue depicts regions with a partial positive charge on the surface, red depicts regions with a partial negative charge, and grey shows neutral areas. **(b)** The predicted conformation depicted as ball-and-stick; no specific interactions are predicted between the ligand and the protein. The adjacent amino acids (< 5 Å), buttressing the ligand, are shown as lines. Hydrogen atoms of the amino acids are omitted for clarity.

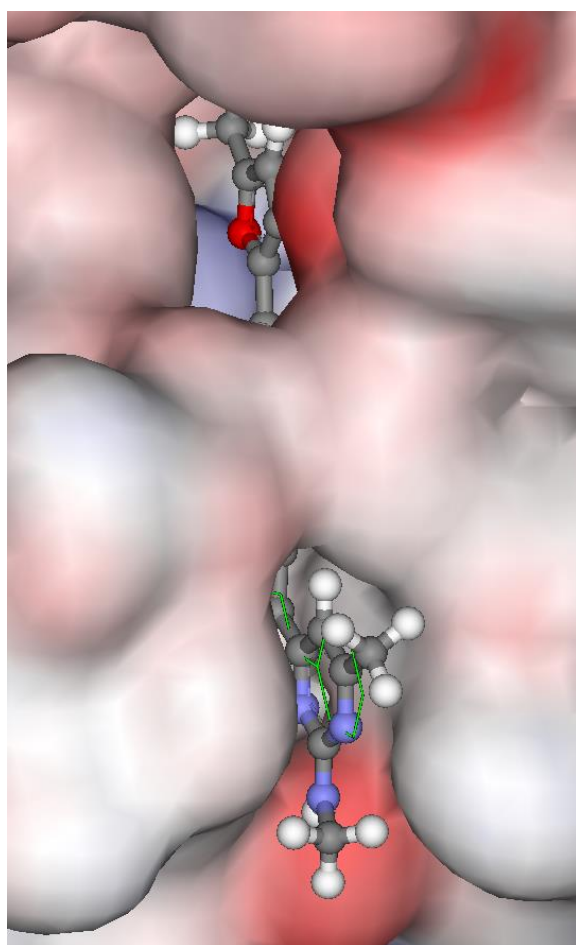


(a)

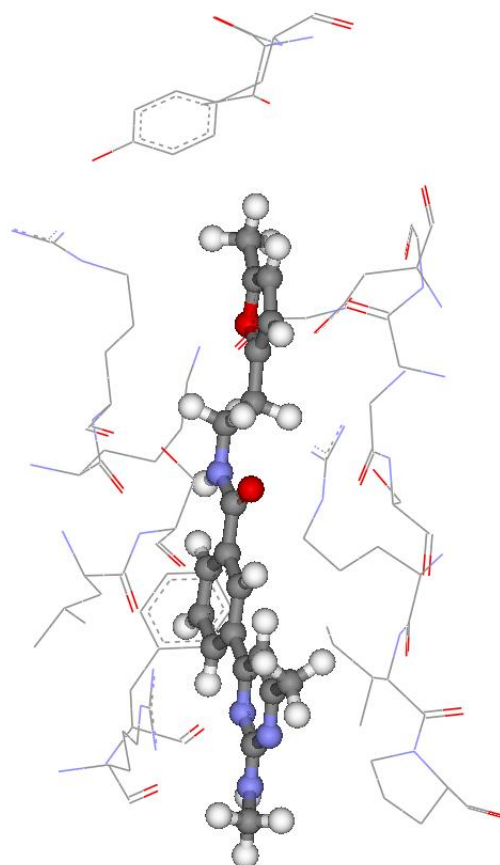


(b)

Supplementary Figure S10. The docked pose of compound **6** in the cryptic pocket of HSP70 as predicted by the ASP scoring function. **(a)** Compound **6** shown as ball-and-stick and the crystallized ligand **1** depicted as green lines. The protein surface shown where blue depicts regions with a partial positive charge on the surface, red depicts regions with a partial negative charge, and grey shows neutral areas. **(b)** The predicted conformation depicted as ball-and-stick; no specific interactions are predicted between the ligand and the protein. The adjacent amino acids ($< 5 \text{ \AA}$), buttressing the ligand, are shown as lines. Hydrogen atoms of the amino acids are omitted for clarity.

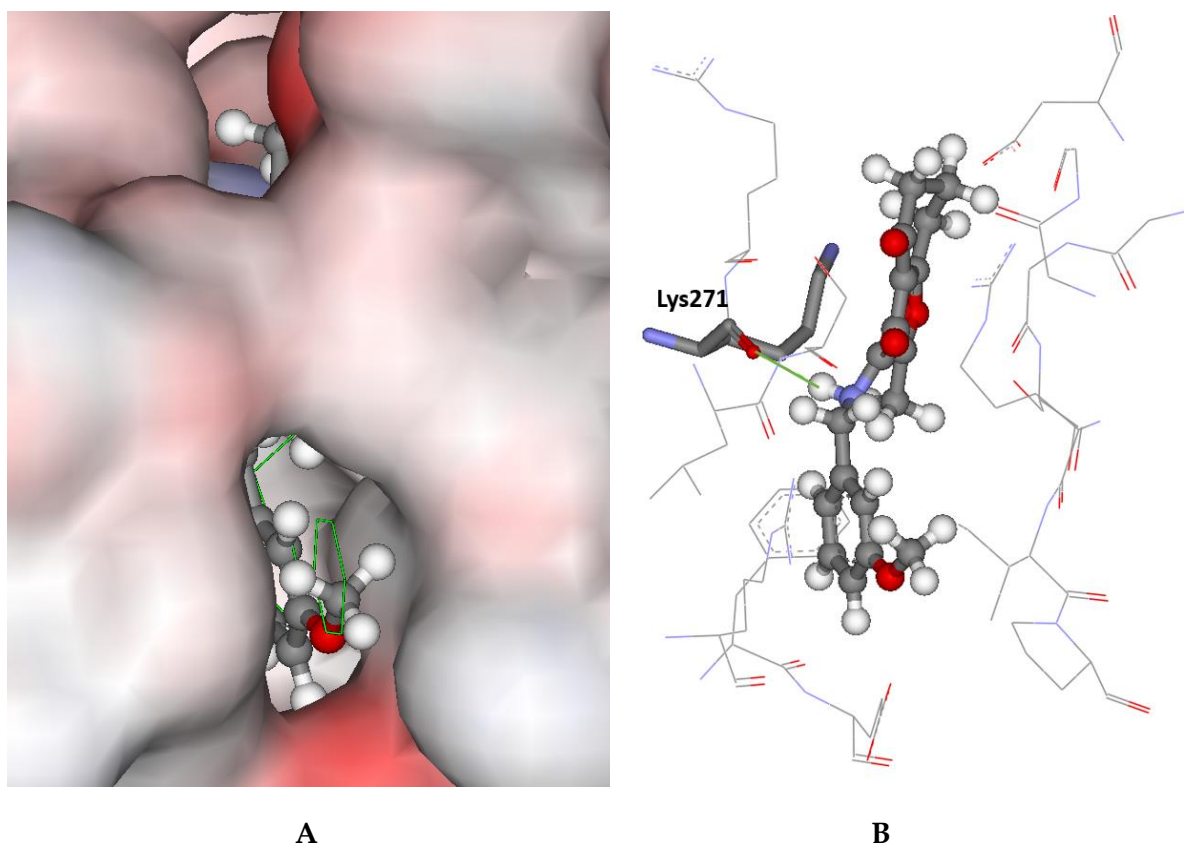


(a)

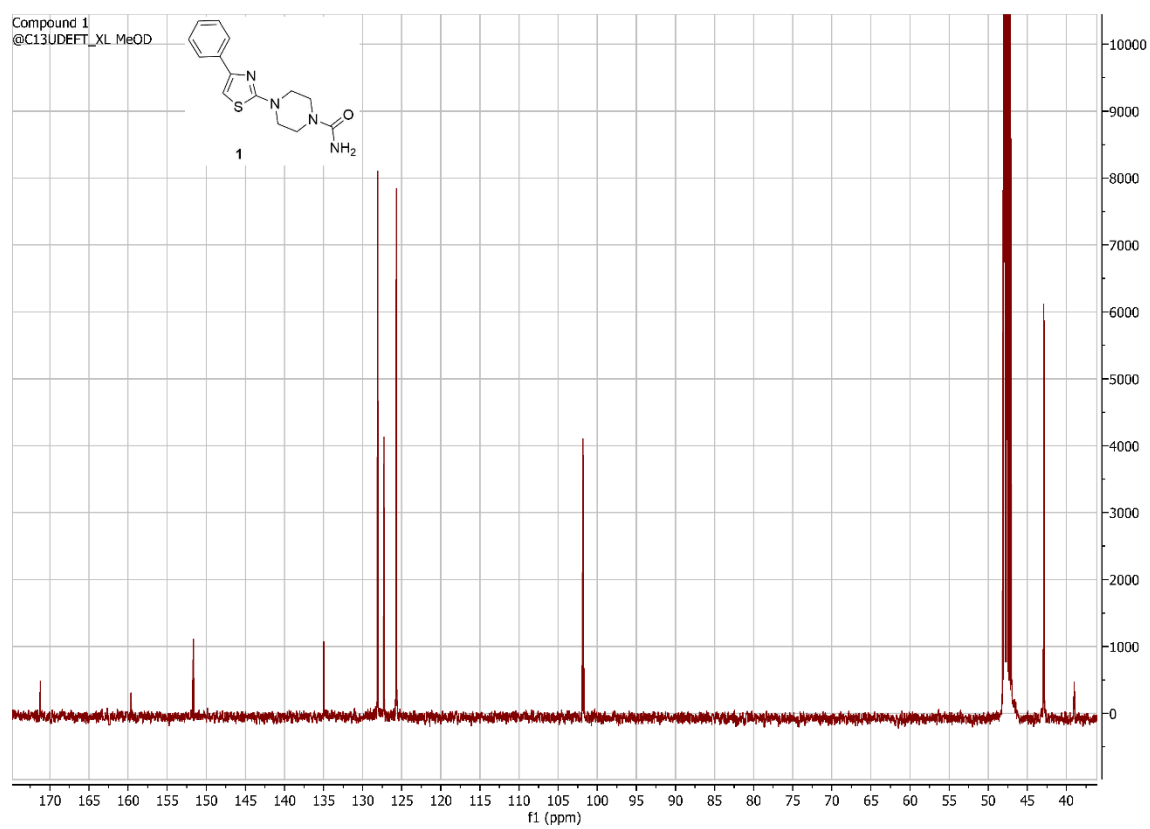
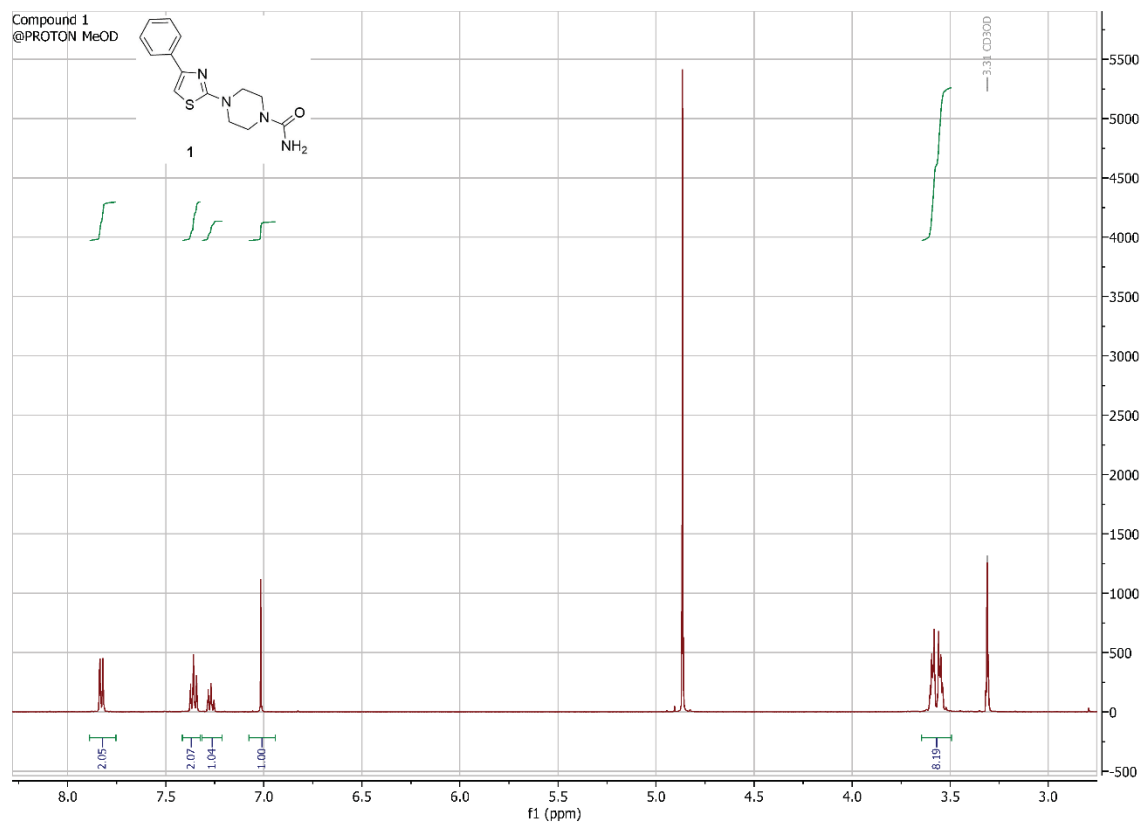


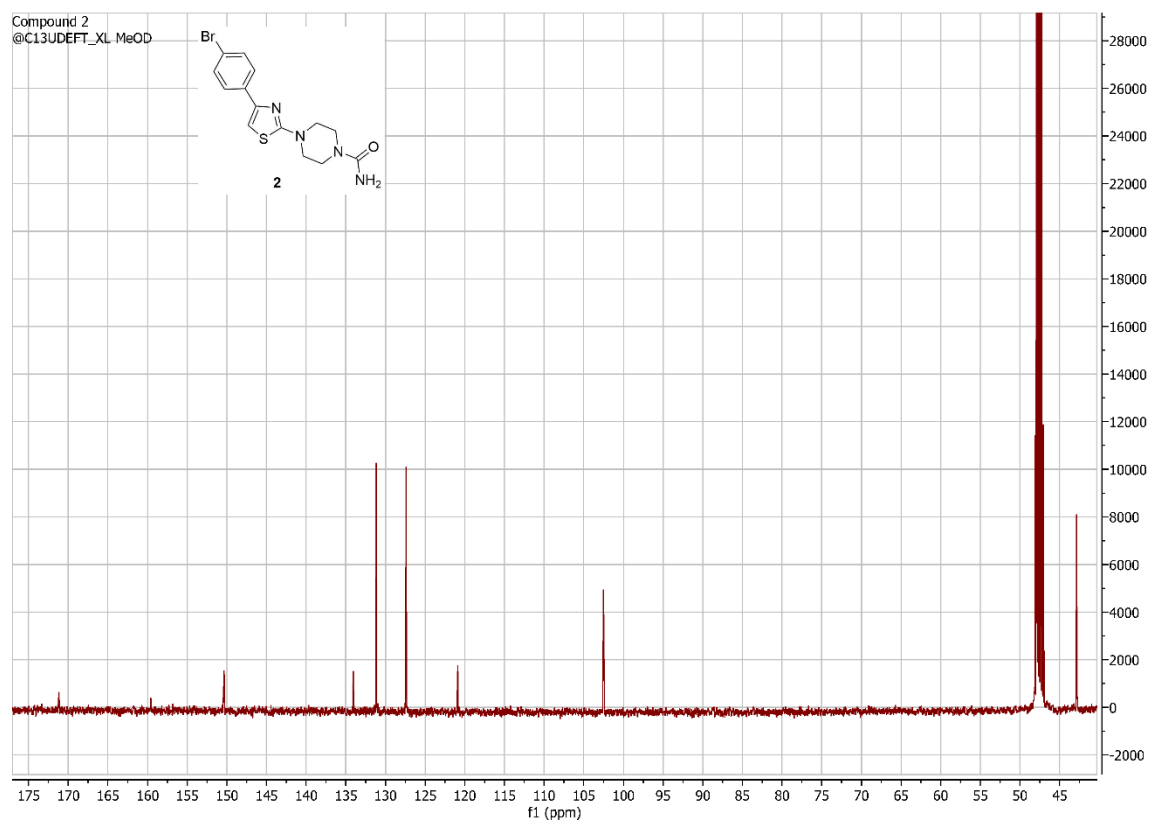
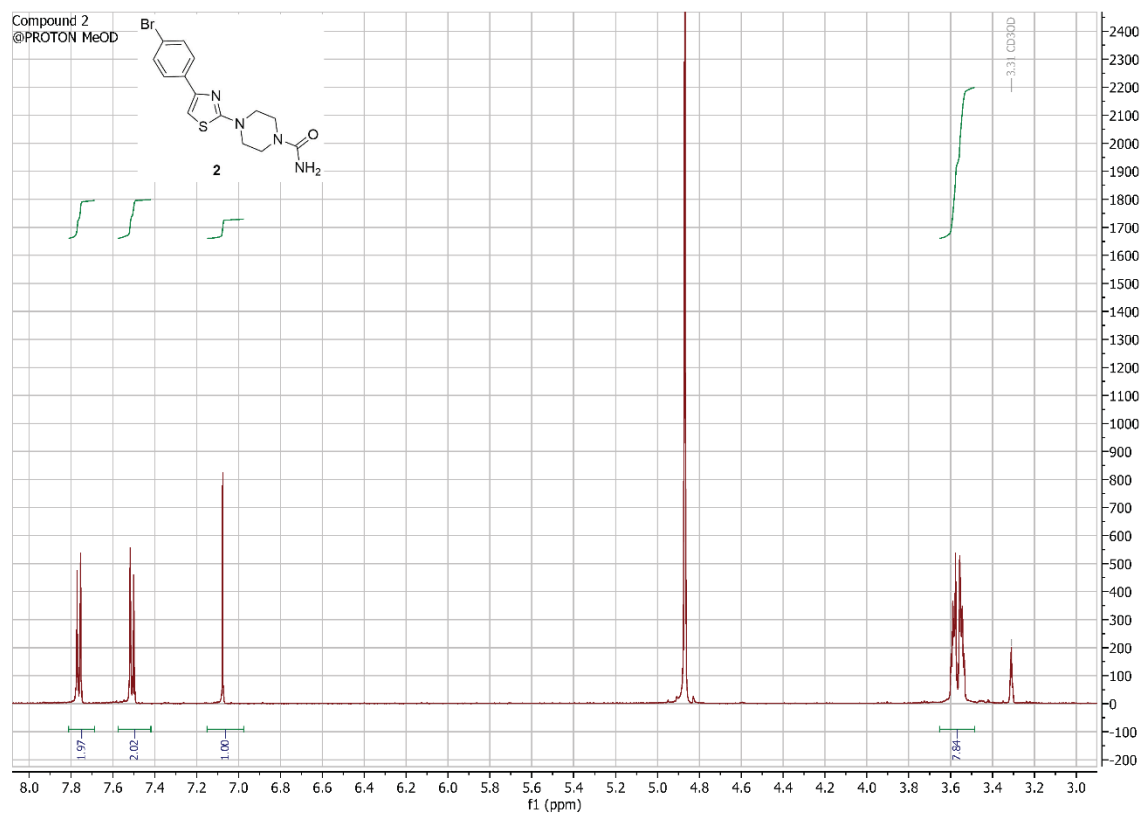
(b)

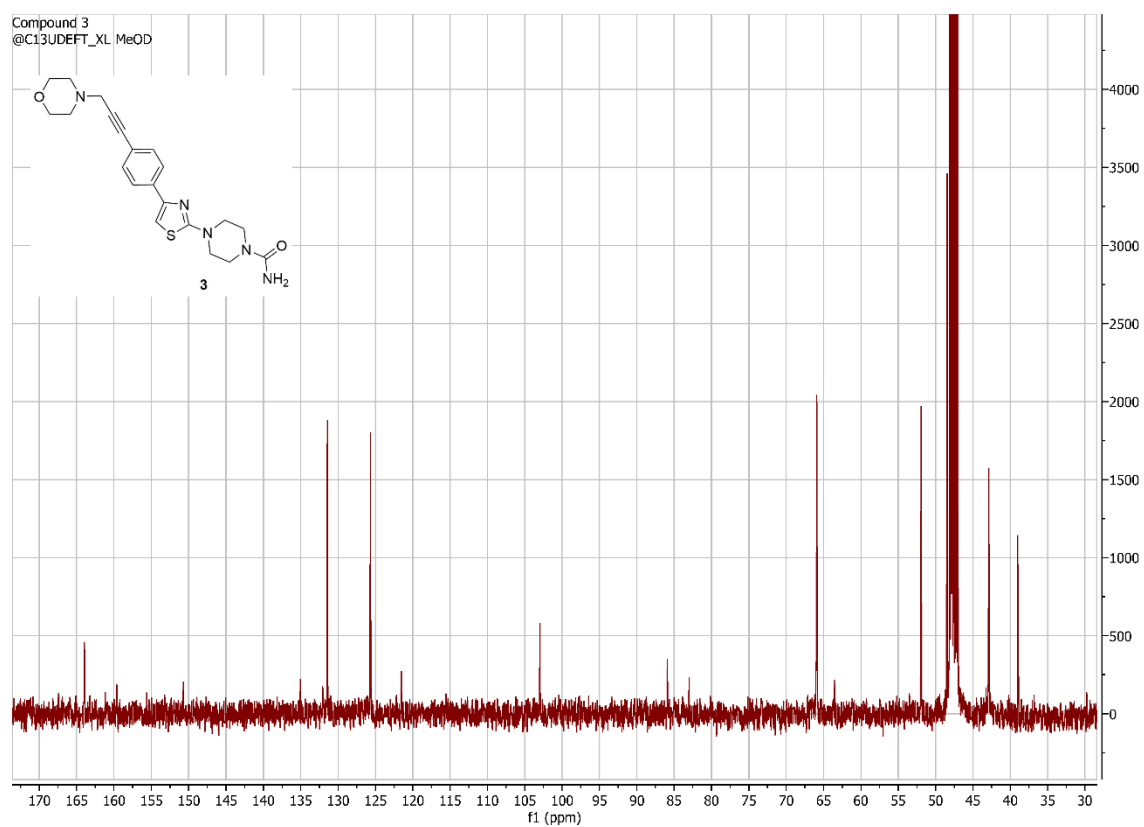
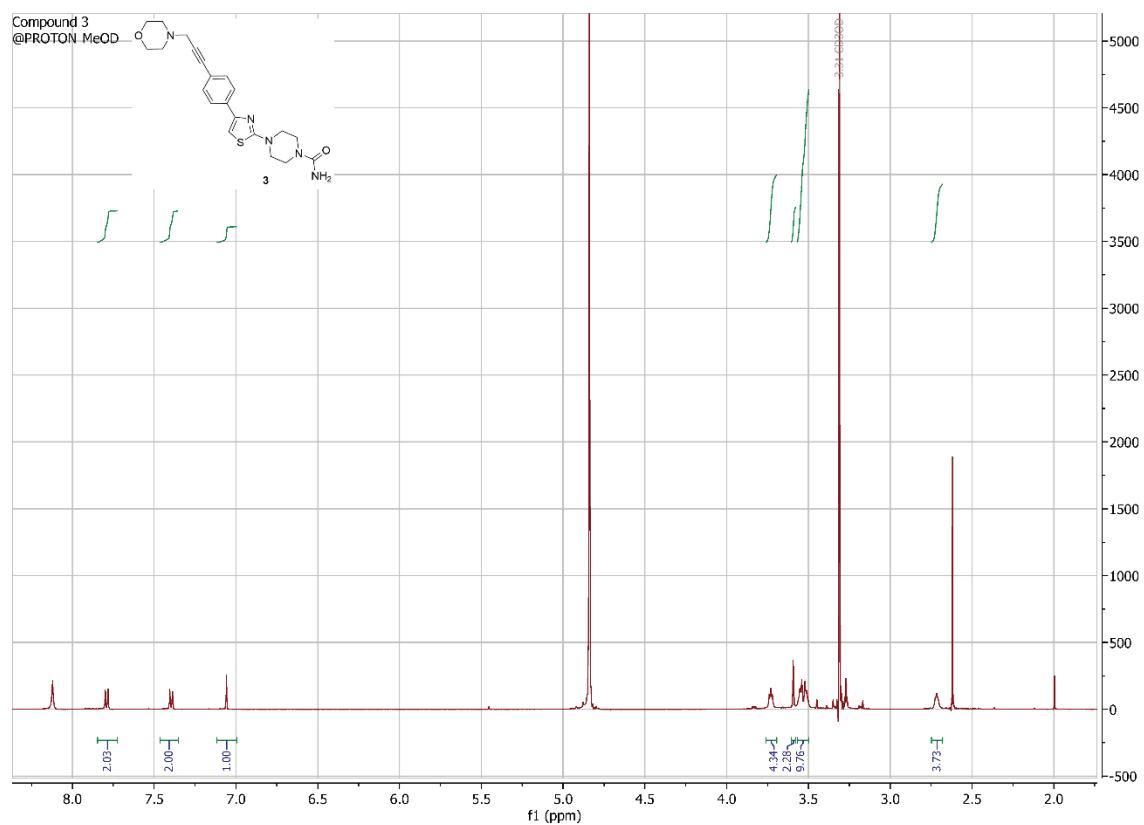
Supplementary Figure S11. The docked pose of compound 7 in the cryptic pocket of HSP70 as predicted by the ASP scoring function. (a) Compound 7 shown as ball-and-stick and the crystallized ligand 1 depicted as green lines. The protein surface shown where blue depicts regions with a partial positive charge on the surface, red depicts regions with a partial negative charge, and grey shows neutral areas. (b) The predicted conformation depicted as ball-and-stick; no specific interactions are predicted between the ligand and the protein. The adjacent amino acids (< 5 Å), buttressing the ligand, are shown as lines. Hydrogen atoms of the amino acids are omitted for clarity.



Supplementary Figure S12. The docked pose of compound **8** in the cryptic pocket of HSP70 as predicted by the ASP scoring function. **(A)** Compound **8** shown as ball-and-stick format and the crystallized ligand **1** depicted as green lines. The protein surface shown where blue depicts regions with a partial positive charge on the surface, red depicts regions with a partial negative charge, and grey shows neutral areas. **(B)** The predicted conformation depicted as ball-and-stick; hydrogen bonding between the Lys271 carbonyl backbone group and the amine moiety in the ligand is seen (green line). The adjacent amino acids ($< 5 \text{ \AA}$), buttressing the ligand, are shown as lines. Hydrogen atoms of the amino acids are omitted for clarity.

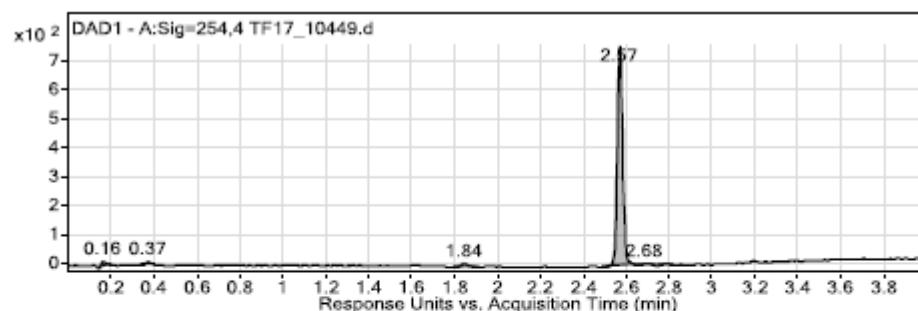






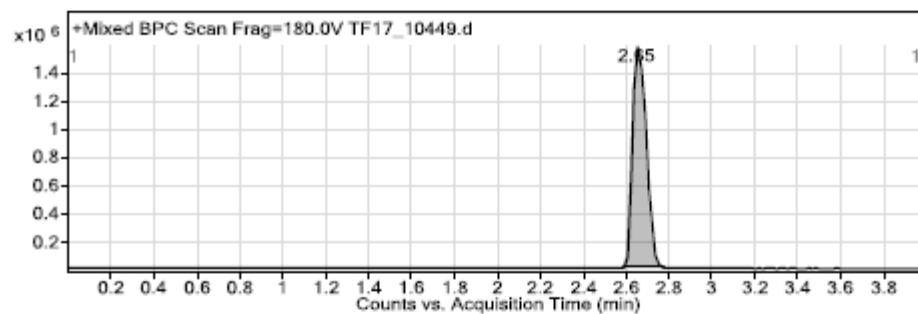
Supplementary Figure S13. ^1H and ^{13}C NMR spectra for compounds 1, 2 and 3.

Compound 1



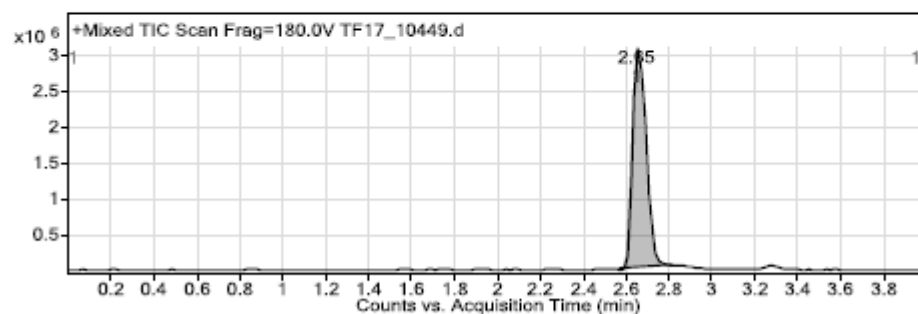
User Chromatogram Peak List

Peak #	RT (mins)	Area	Area Sum %
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3	1.84	34.56	2.39
4	2.57	1328.87	91.85
5	2.68	15.85	1.1
6	2.78	14.65	1.01



User Chromatogram Peak List

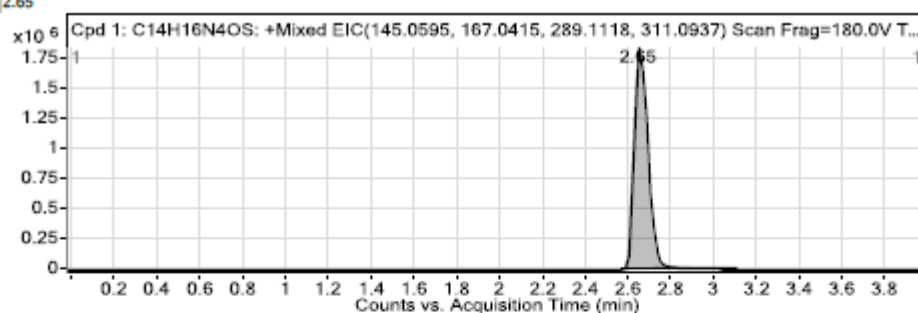
Peak #	RT (mins)	Area	Area Sum %	Base Peak m/z
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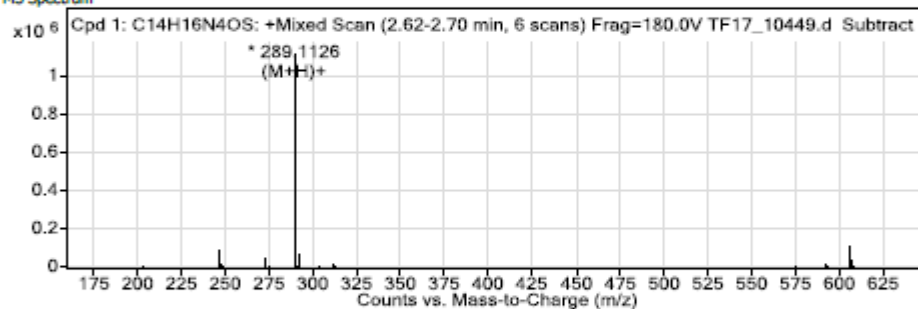
User Chromatogram Peak List

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RT
2.65



MS Spectrum

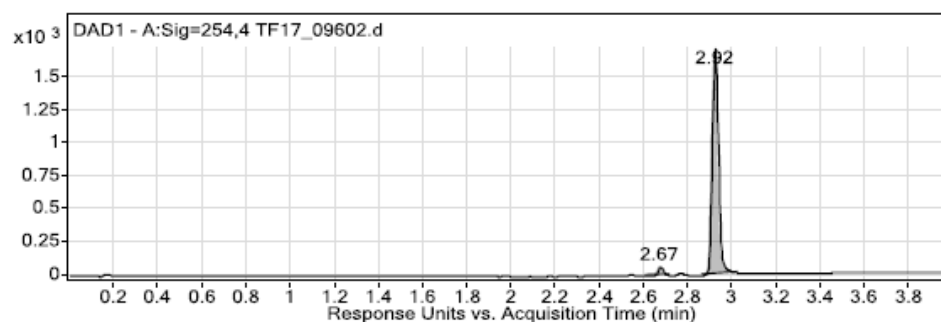


MS Spectrum Peak List

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289.3229				110781
289.4331				6723
289.5367				11208
290.1156	C ₁₄ H ₁₇ N ₄ O ₅	290.1144	3.85	278287
290.3276				13527
291.1124	C ₁₄ H ₁₇ N ₄ O ₅	291.1101	8.03	63929
292.114	C ₁₄ H ₁₇ N ₄ O ₅	292.1116	8.44	9729
311.0936	C ₁₄ H ₁₆ N ₄ NaO ₅	311.0937	-0.43	22912
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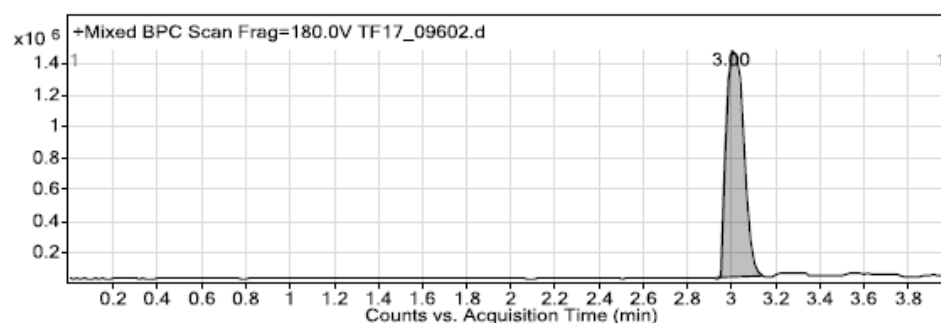
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Compound 2



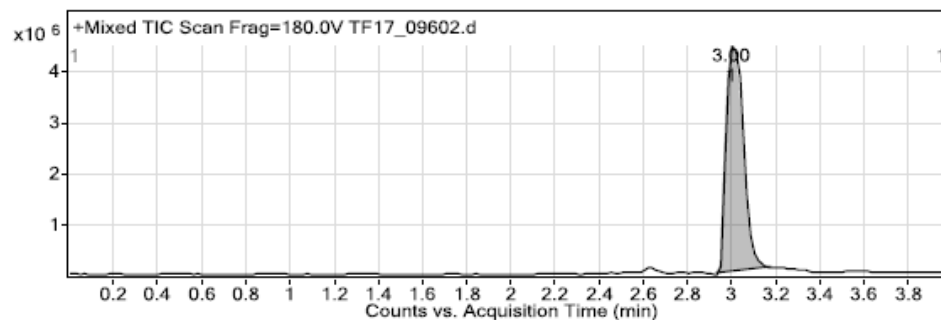
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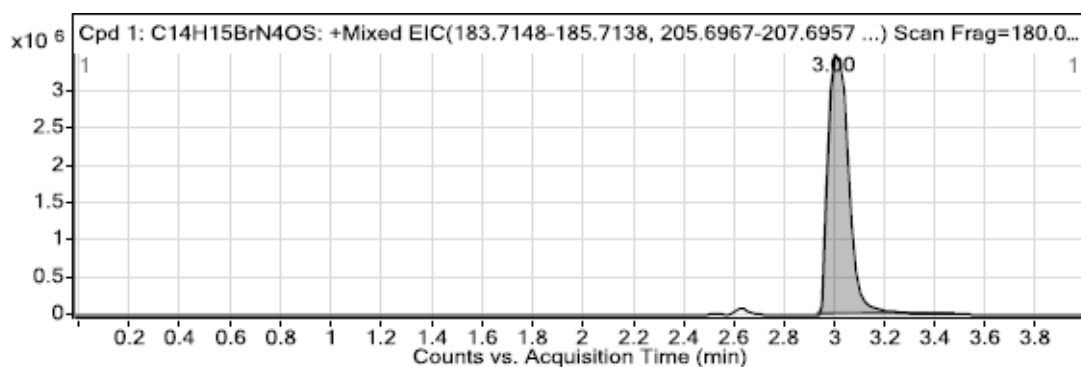
User Chromatogram Peak List

Peak #	RT (mins)	Area	Area Sum %	Base Peak m/z
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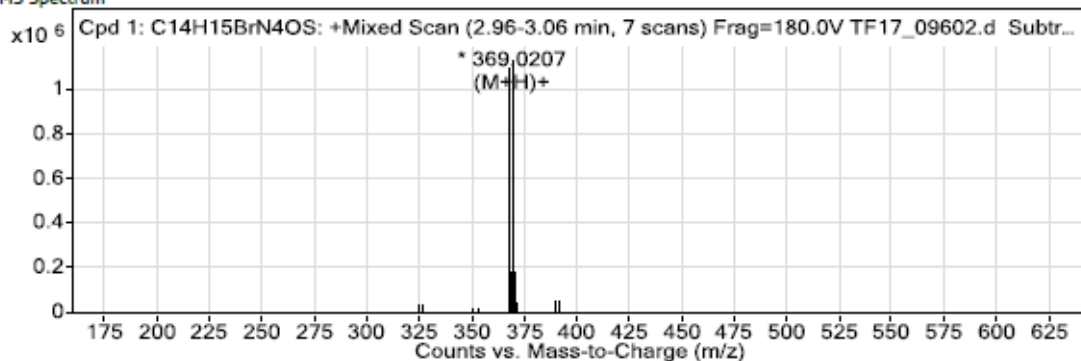


User Chromatogram Peak List

Peak #	RT (mins)	Area	Area Sum %	Base Peak m/z
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MS Spectrum

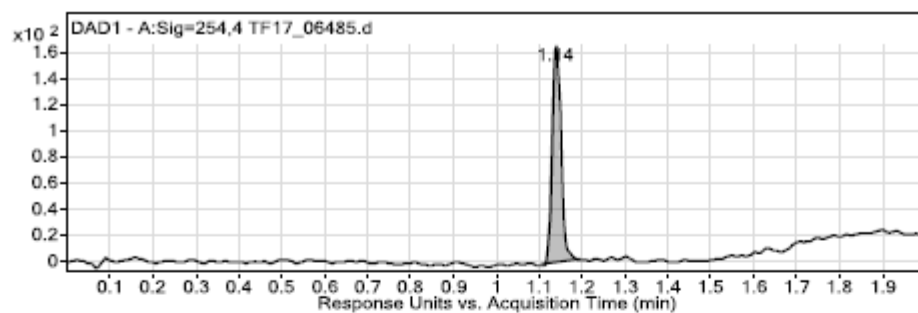


MS Spectrum Peak List

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367.2595				94116
368.0245	C ₁₄ H ₁₆ Br N ₄ O ₅	368.025	-1.19	182806
369.0207	C ₁₄ H ₁₆ Br N ₄ O ₅	369.0202	1.15	1127631
369.257				112090
369.4974				13494
370.0225	C ₁₄ H ₁₆ Br N ₄ O ₅	370.0229	-0.89	181124
371.0186	C ₁₄ H ₁₆ Br N ₄ O ₅	371.0186	-0.14	49207
389.004	C ₁₄ H ₁₅ Br N ₄ Na O ₅	389.0042	-0.59	54675
391.0016	C ₁₄ H ₁₅ Br N ₄ Na O ₅	391.0022	-1.64	57026

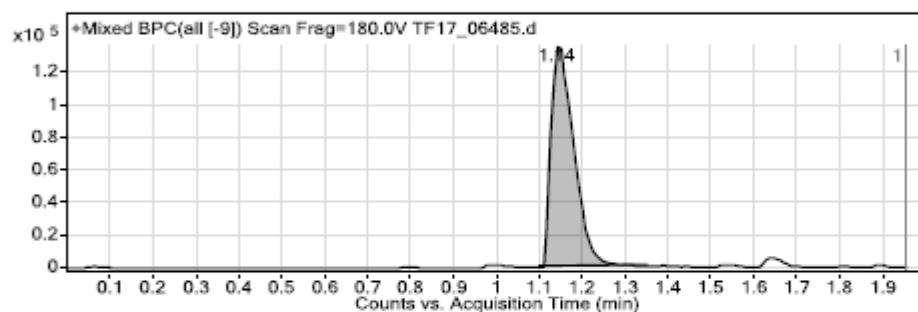
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Compound 3



User Chromatogram Peak List

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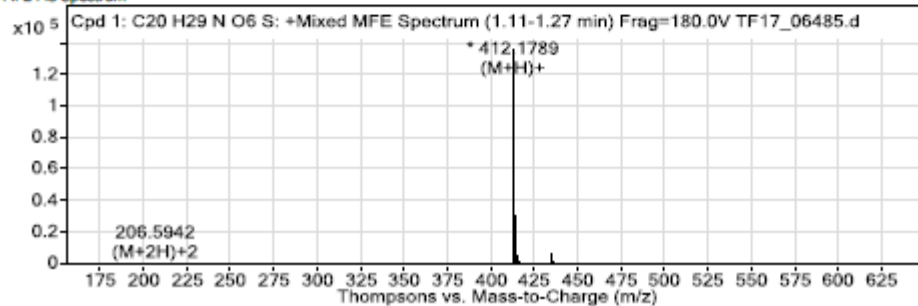


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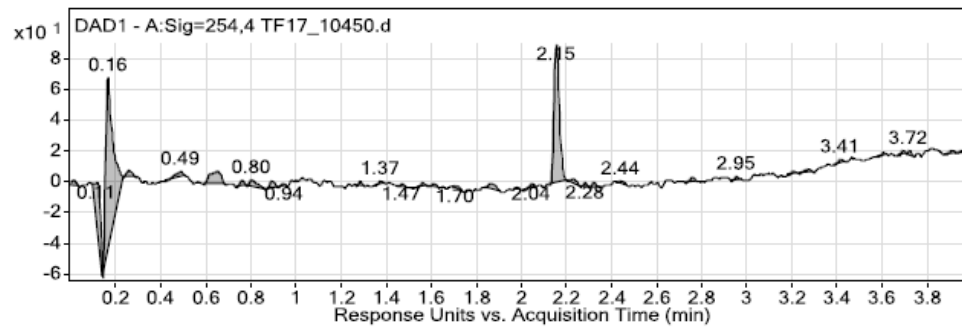
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RT
1.15

MFE MS Spectrum

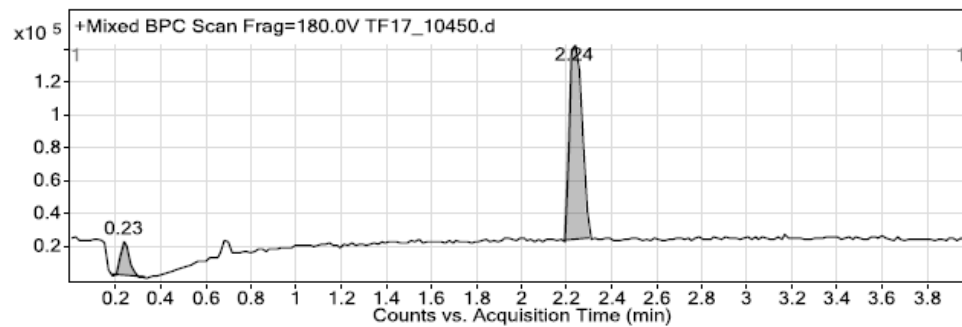


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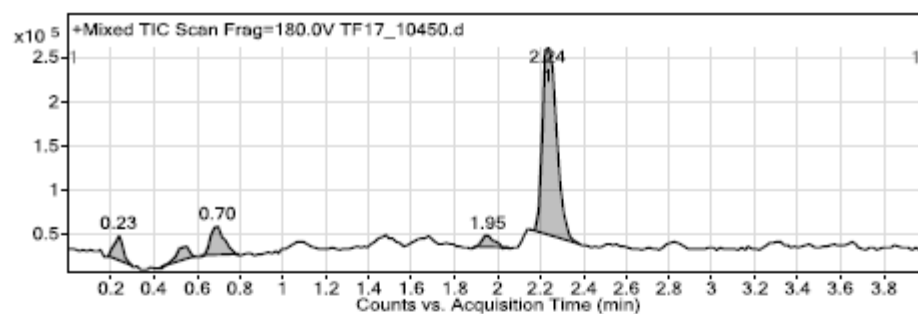
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5	0.49	10.57	1.69
6	0.65	29.58	4.72
7	0.76	5.35	0.85
8	0.8	12.25	1.95
9	0.89	8.66	1.38
17	1.86	8.75	1.4
19	2.15	147.26	23.49



User Chromatogram Peak List

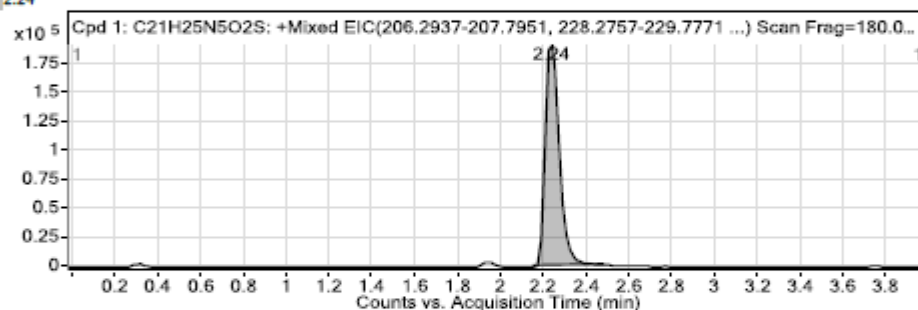
Peak #	RT (mins)	Area	Area Sum %	Base Peak m/z
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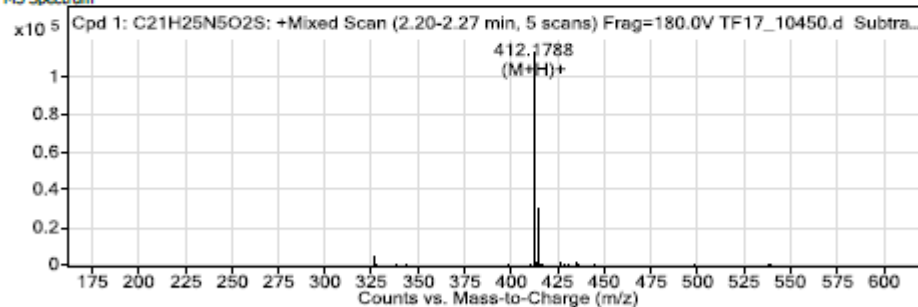
User Chromatogram Peak List

Peak #	RT (mins)	Area	Area Sum %	Base Peak m/z
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3	0.7	153066	11.23	306.154
4	1.95	60551	4.44	195.0892
5	2.24	982115	72.08	412.1801

RT
2.24



MS Spectrum

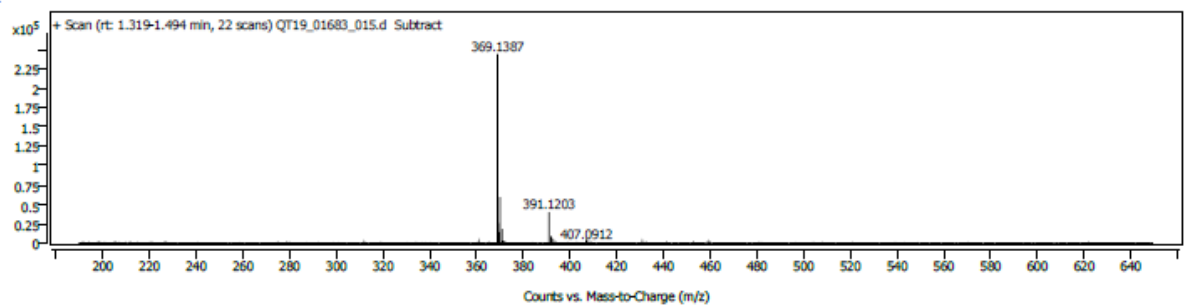
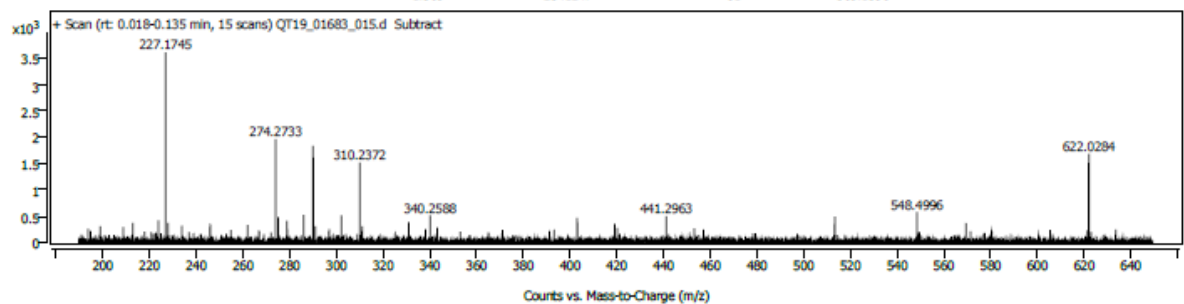
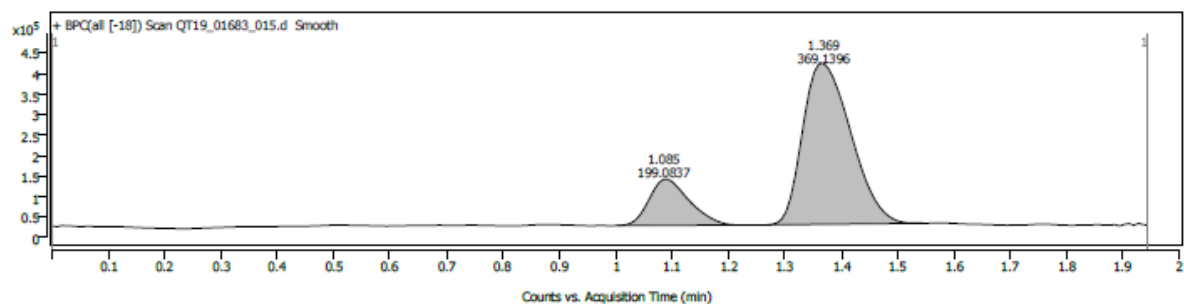
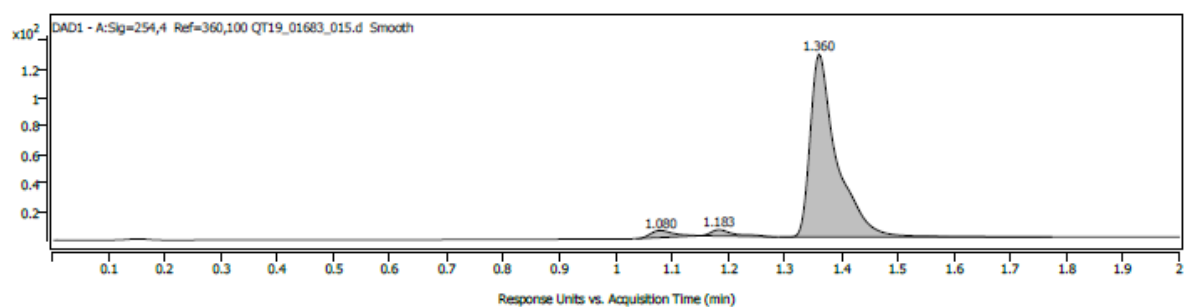


MS Spectrum Peak List

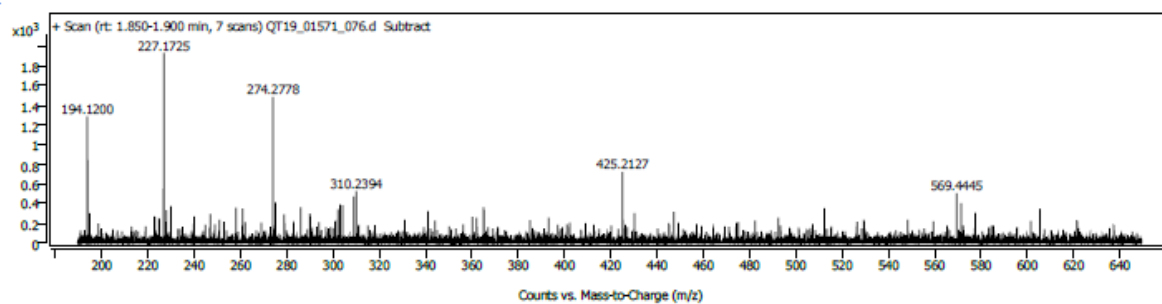
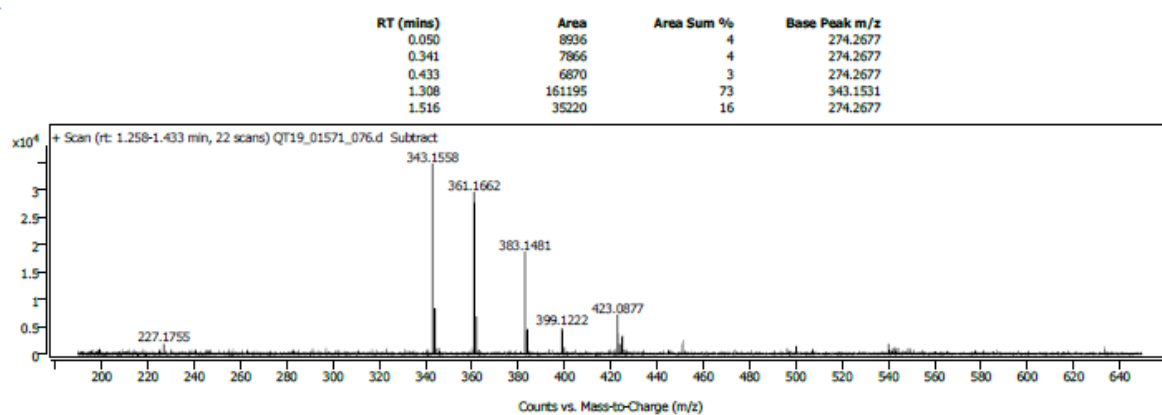
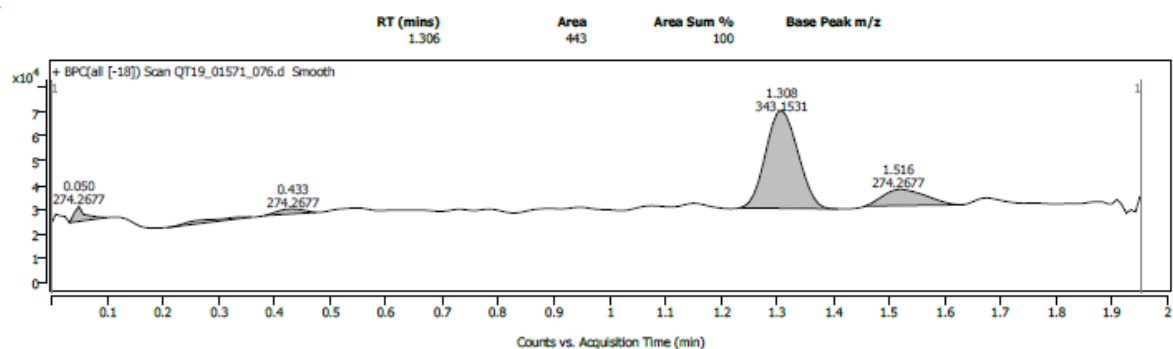
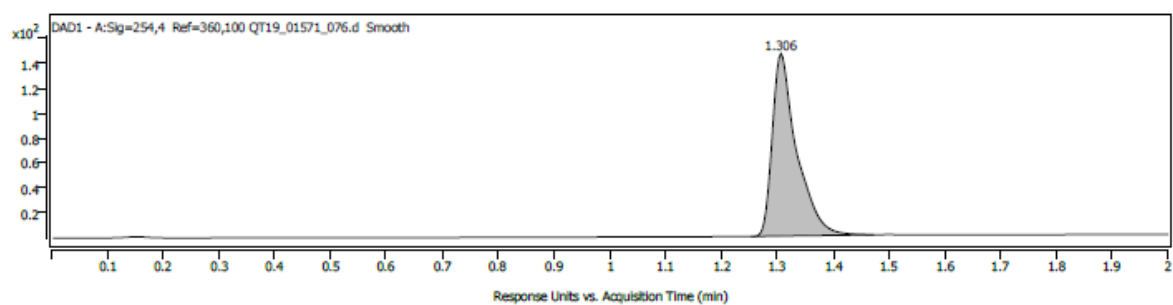
Observed m/z	Calculated Formula [M+H] ⁺	Target m/z	Error (ppm)	Abundance
410.164				1090
411.1669				479
412.1788	C ₂₁ H ₂₆ N ₅ O ₂ S	412.1802	-3.21	113857
412.4315				2137
413.1831	C ₂₁ H ₂₆ N ₅ O ₂ S	413.183	0.29	30286
413.4381				548
414.182	C ₂₁ H ₂₆ N ₅ O ₂ S	414.1803	4.08	7243
415.1834	C ₂₁ H ₂₆ N ₅ O ₂ S	415.1811	5.34	1465
434.1612	C ₂₁ H ₂₅ N ₅ NaO ₂ S	434.1621	-2.04	2307
435.1642	C ₂₁ H ₂₅ N ₅ NaO ₂ S	435.1649	-1.71	741

--- End Of Report ---

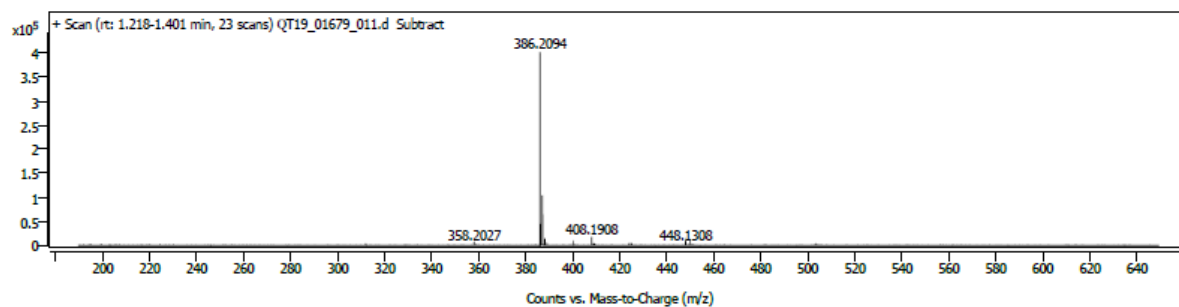
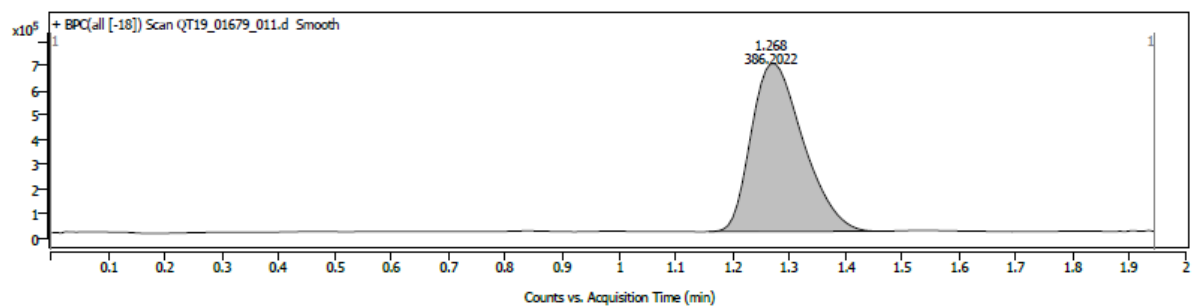
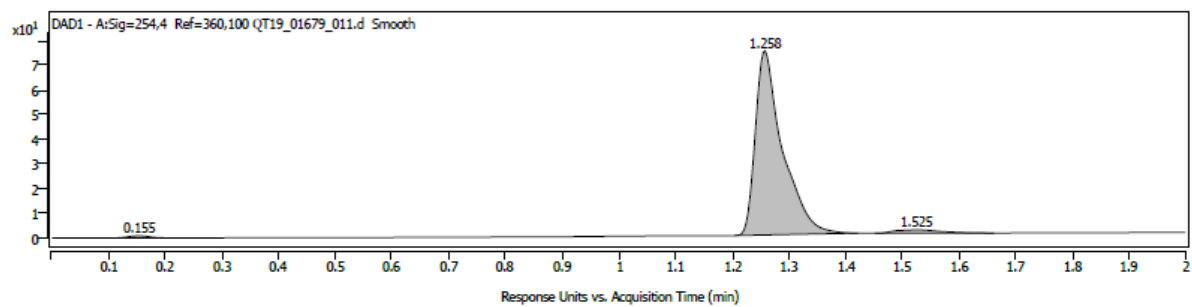
Compound 4



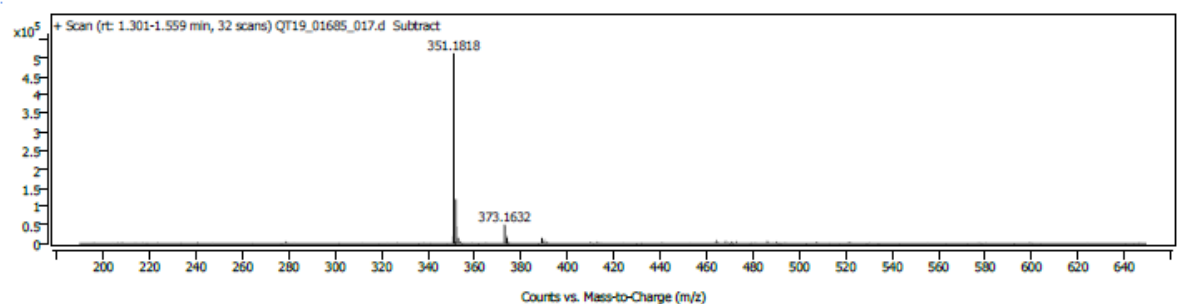
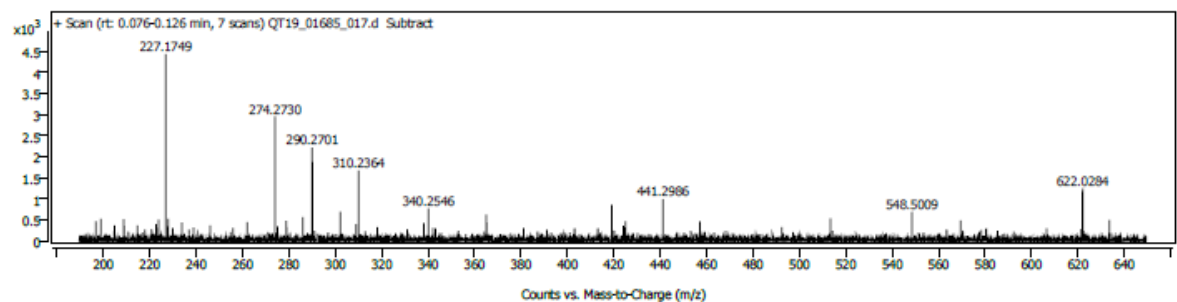
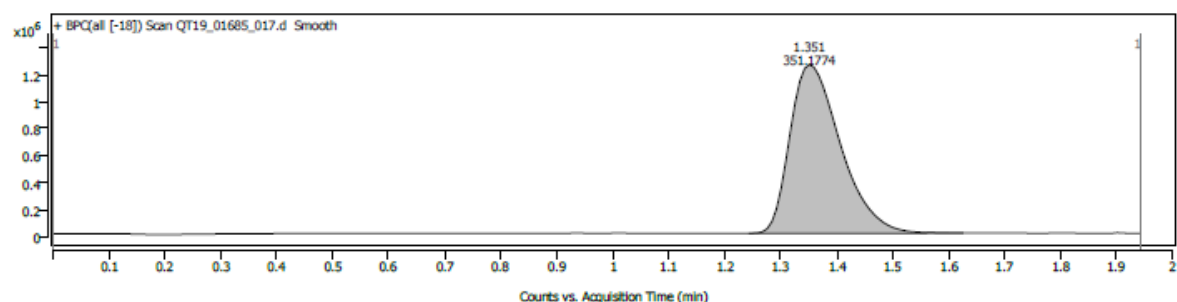
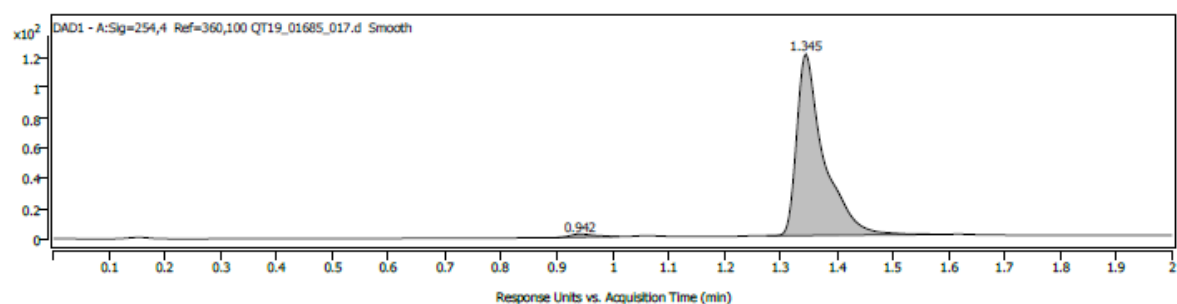
Compound 5



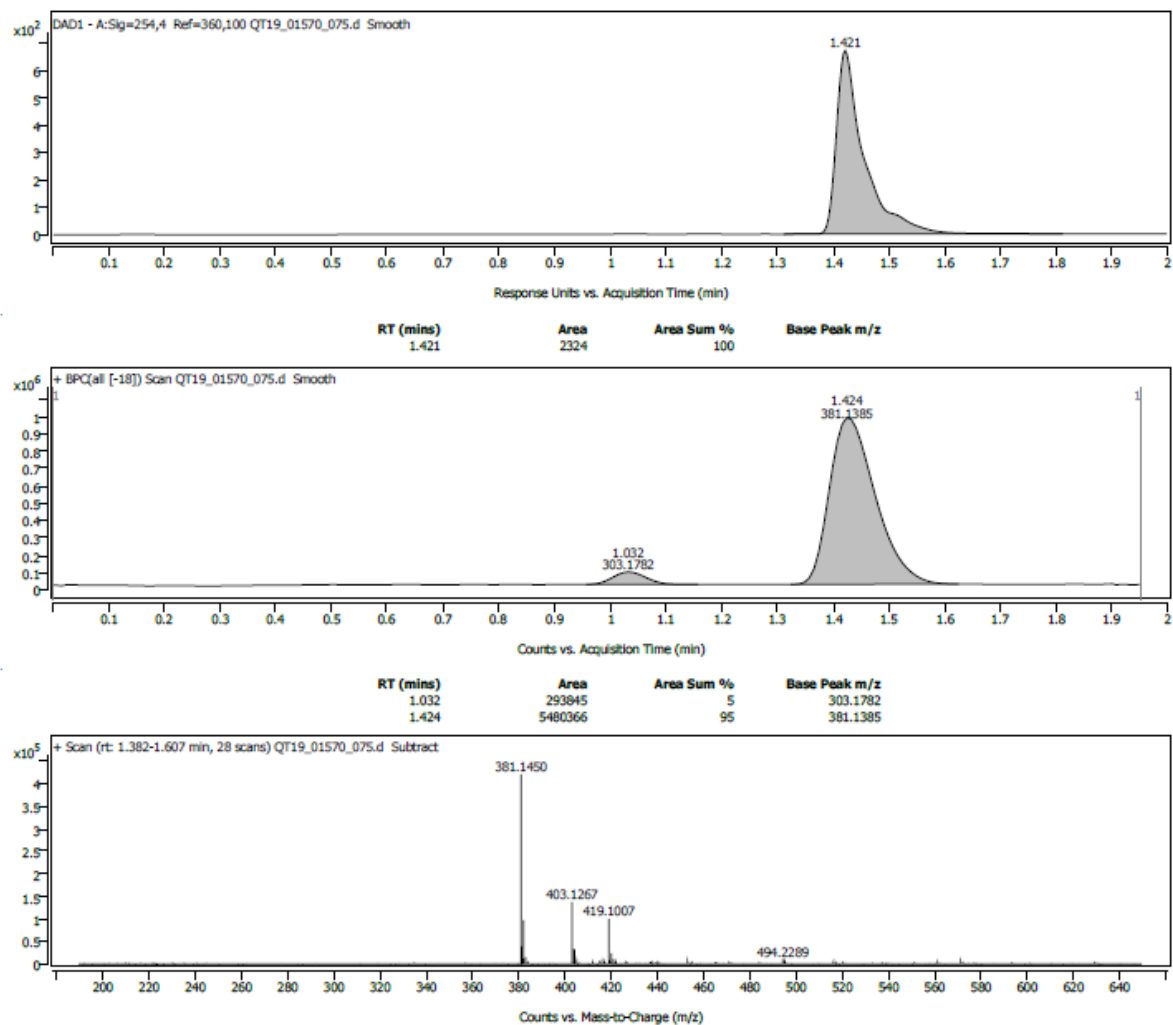
Compound 6



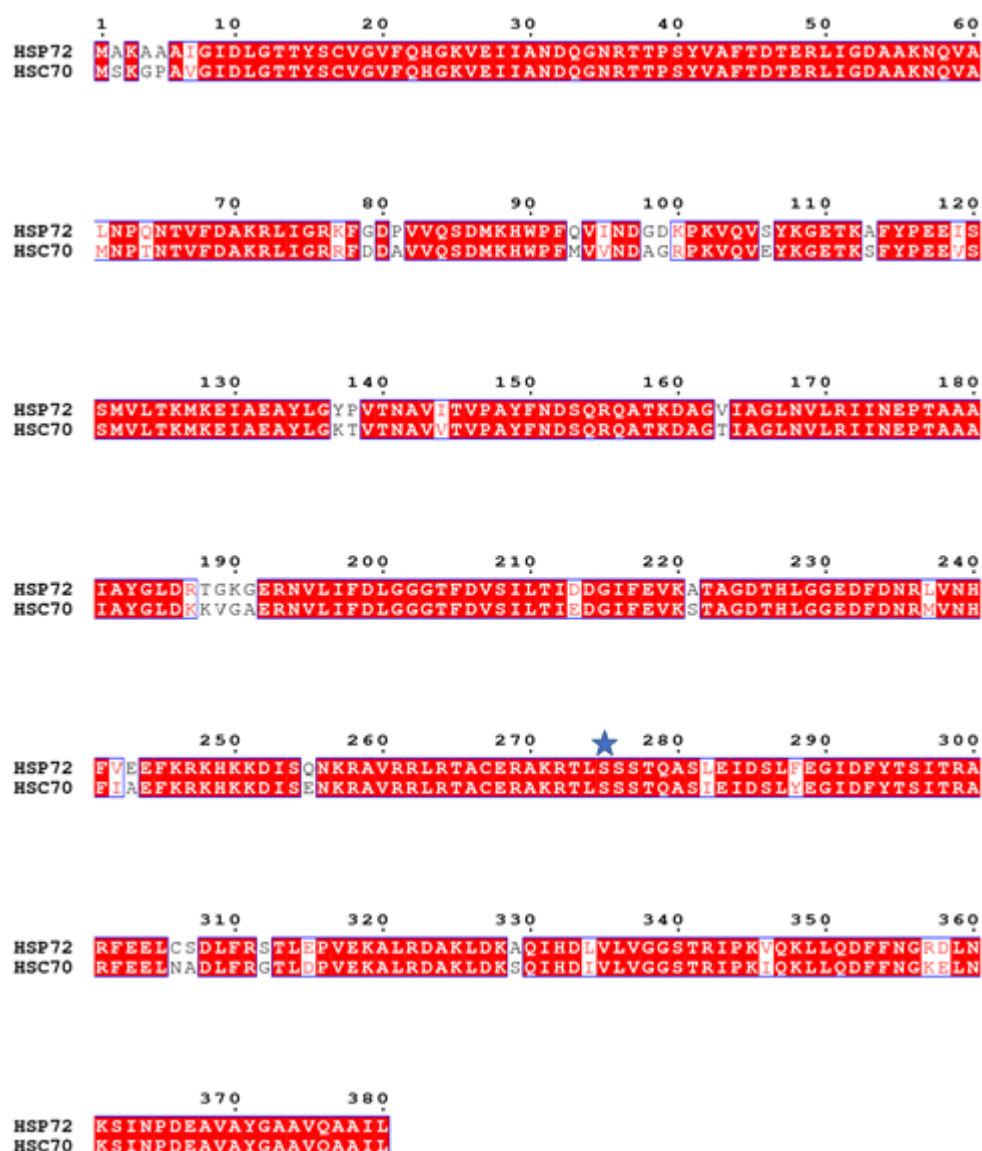
Compound 7



Compound 8



Supplementary Figure S14: LC-MS spectra for compounds 1 – 8.

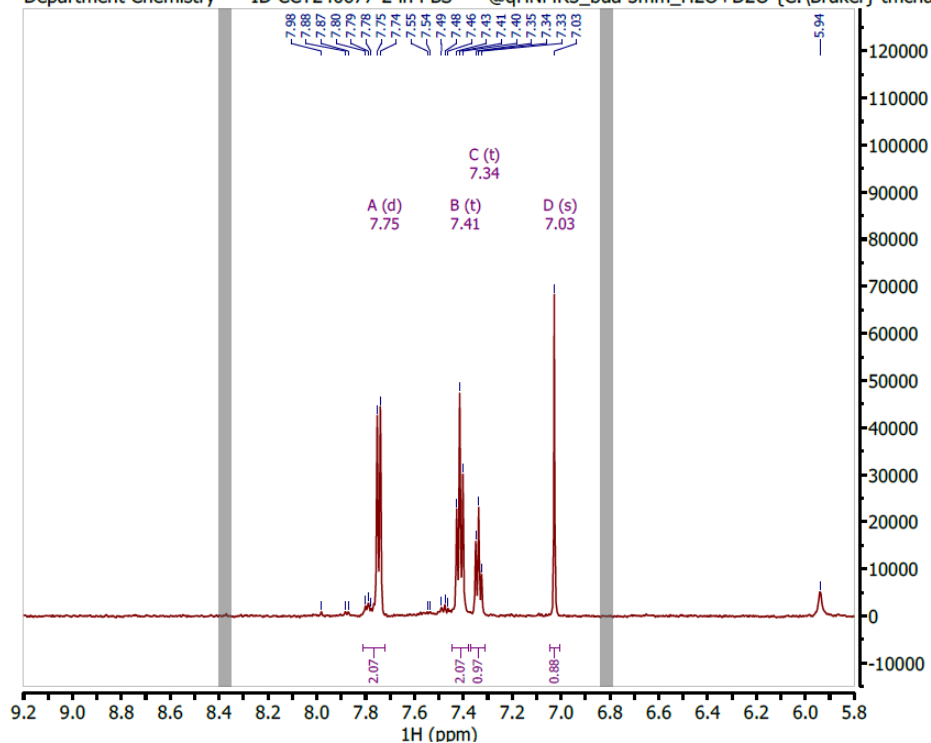


Supplementary Figure S15: Sequence alignment of the nucleotide binding domains of human HSP72 and HSC70 proteins. Identical residues are shown in white font on a red background and similar residues in red font on a white background. Residue S275, crucial for the binding of the adenine ring of ATP, is highlighted with a blue star. Sequence identity is 85.1 % on the full-length proteins, rising to 88.7 % for the nucleotide binding domains only (residues 1 to 380 of both proteins), and to 100 % for the residues coating the ATP binding site and/or the binding site of compound **1** (residues 201-204, 229-231, 267-276, 299-303, 339-345 and 366). The sequence alignment was performed with Clustal Omega and its visual representation with ESript 3.0.

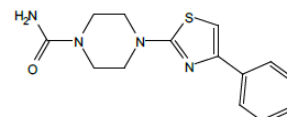
Multiplet	NN	Range	Abs. Integral	Concentration	SNR
A(d)	2	7.81..7.72	6942472.5126	297.2180	1012.0886
B(t)	2	7.44..7.38	6939013.8658	297.0699	1076.0009
C(t)	1	7.37..7.31	3259444.4027	279.0837	525.9891

Concentration Average: 291.1239 uM
RMSD%: 2.9245

Department Chemistry — ID CCT240677-2 in PBS — @qHNMR3_bua 3mm_H2O+D2O {C:\Bruker} tmchar



Parameter	Value
Title	TFFM-5208-005-CCT240677-2.10.fid
Solvent	3mm_H2O+D2O
Temperature	298.0
Pulse Sequence	ic1pmpgpf2
Probe	Z75812_0002 (CP TCI 60053 H-C/ N-D-05 Z)
Number of Scans	32
Receiver Gain	101.0
Relaxation Delay	20.0000
Acquisition Date	2019-06-22T15:12:21
Spectrometer Frequency	600.14



Supplementary Figure S16: Determination of pH 7.4 phosphate buffer solubility of **1** by quantitative NMR.

Supplementary Table S1. Observed changes in NMR signal intensities for **3** on addition of HSP72-NBD and ATP.¹

Conditions	Peak a	Peak b	Peak c
3 + HSP72-NBD	41%	36%	31%
3 + HSP72-NBD + ATP	5%	22%	7%

¹ Quantified from the data shown in Supplementary Figure S4 using MestreNova

Supplementary Table S2. LC-MS data to confirm the identity and purity of vHTS hits **4 – 8**.

Compound	Mass detected	Purity (UV)
4	369.1 (M+H) ⁺	94%
5	361.2 (M+H) ⁺	100%
6	386.2 (M+H) ⁺	96%
7	351.2 (M+H) ⁺	98%
8	381.1 (M+H) ⁺	100%

Supplementary Table S3: Crystallographic data collection and refinement statistics for **1**-HSP72-NBD.

Protein construct	HSP72 1-380
Ligand	1
PDB code	7Q4R
<i>Crystal</i>	
Space group	P 2 ₁ 2 ₁ 2 ₁
Unit cell dimensions (a/b/c in Å)	47.47/78.47/93.50
Unit cell angles (α/β/γ in °)	90/90/90
<i>Data collection and processing</i>	
Beamline	DLS I24
Wavelength (Å)	0.9686
Integration program	XDS
Reduction program	AIMLESS
Resolution range	47.47 – 1.79
Number of unique reflections ^a	32590 (1470)
Completeness ^a	96.8 (75)
Redundancy ^a	5.5 (3.4)
R _{merge} (%) ^a	5.9 (37.7)
I/σ(I) ^a	15.7 (2.2)
CC _{1/2} ^{a, b}	0.999 (0.864)
<i>Refinement</i>	
Program	BUSTER
R _{work} (%)	19.17
R _{free} (%)	23.49
Number of residues	383
Number of water molecules	409
Average B-factor (Å ²)	26.30
Ramachandran favoured (%)	100
Ramachandran outliers (%)	0
RMSD bonds (Å)	0.012
RMSD angles (°)	1.496

^a Values in parentheses are for the highest resolution shell; ^b Half-dataset correlation coefficient [1].

Reference

1. Karplus, P.A.; Diederichs, K. Linking crystallographic model and data quality. *Science* **2012**, *336*, 1030–1033. <https://doi.org/10.1126/science.1218231>.