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Supplemental Experimental Procedures

Supplemental References

Figure S1

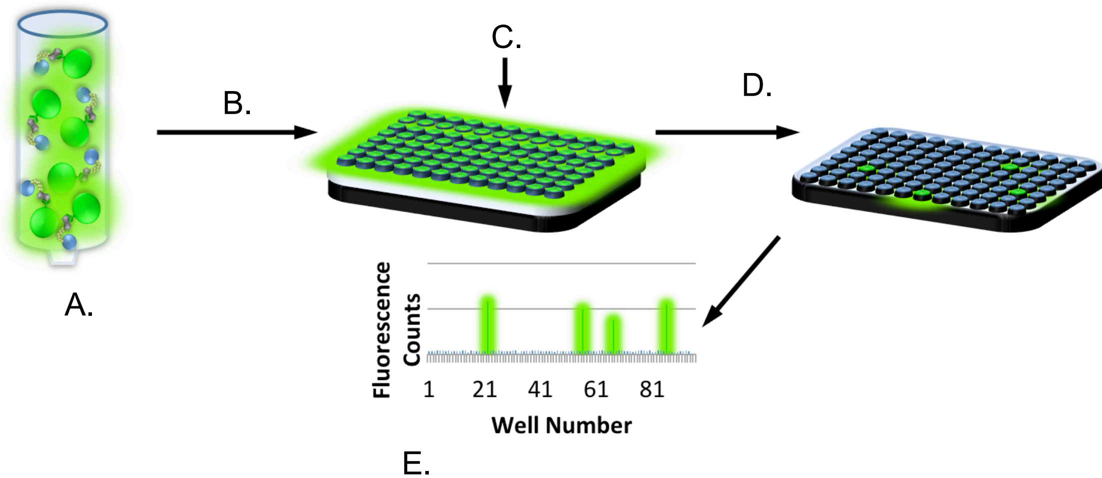
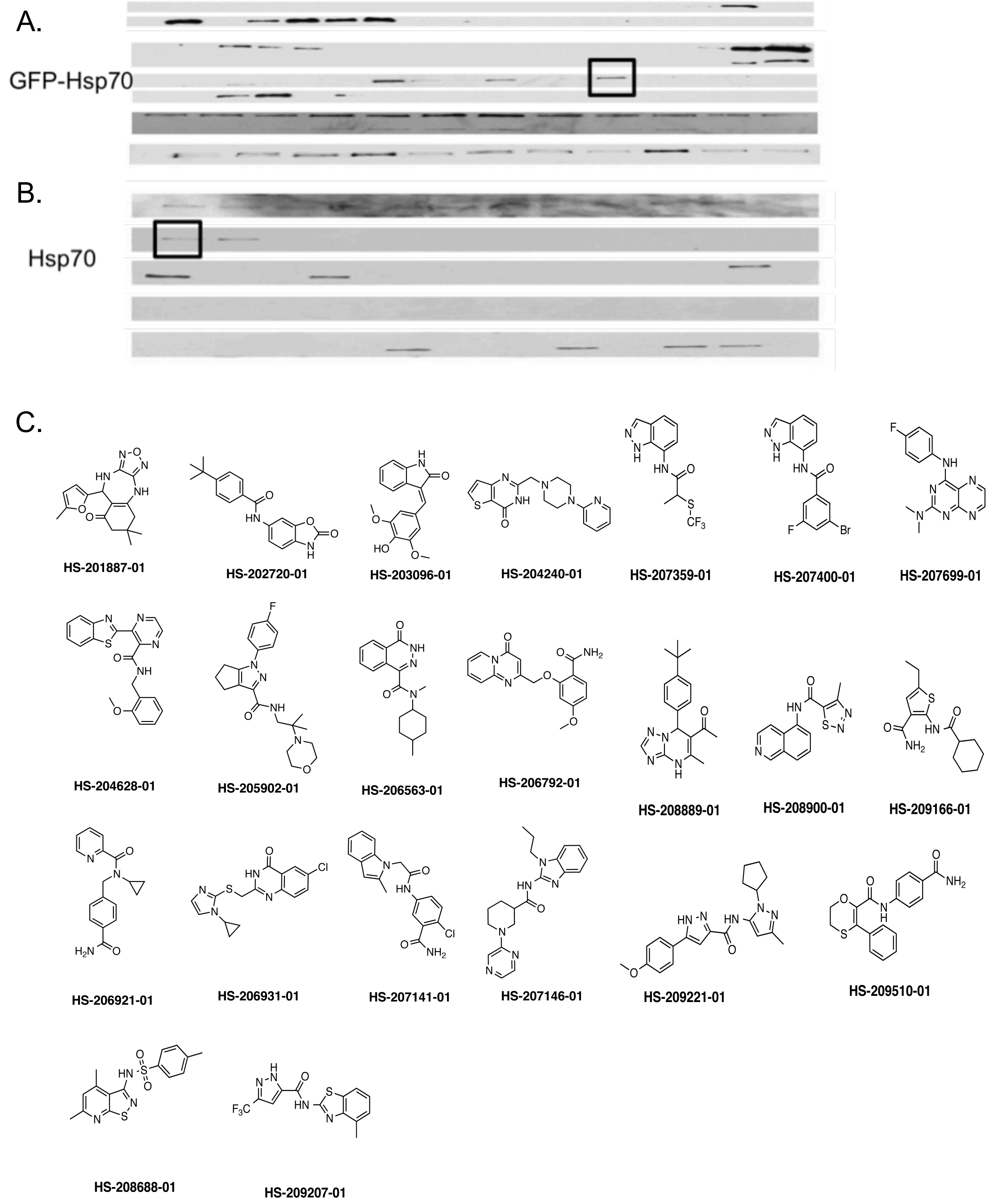
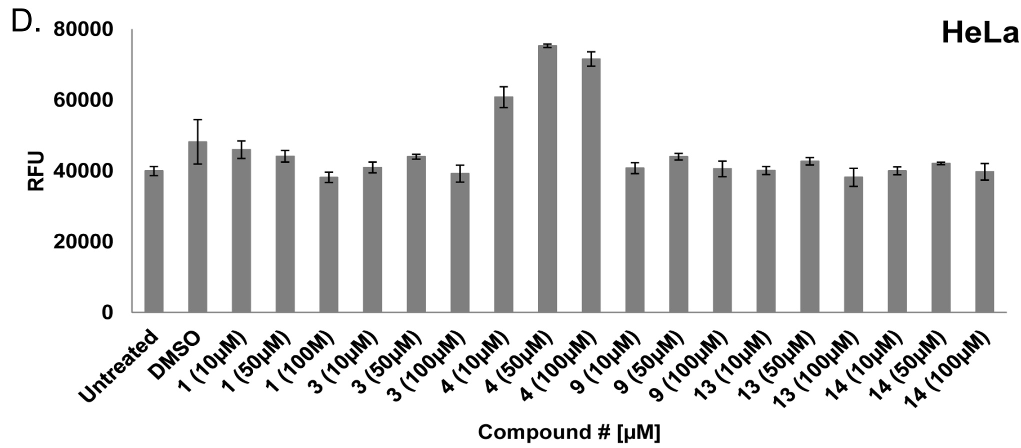
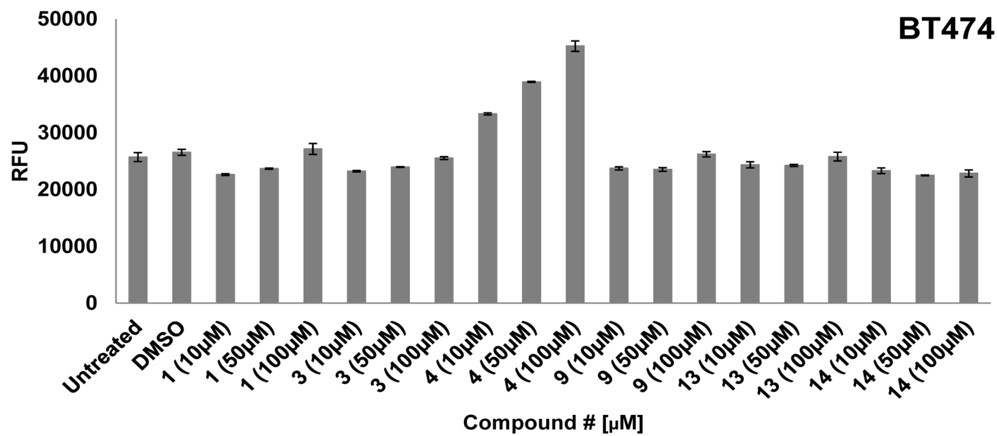


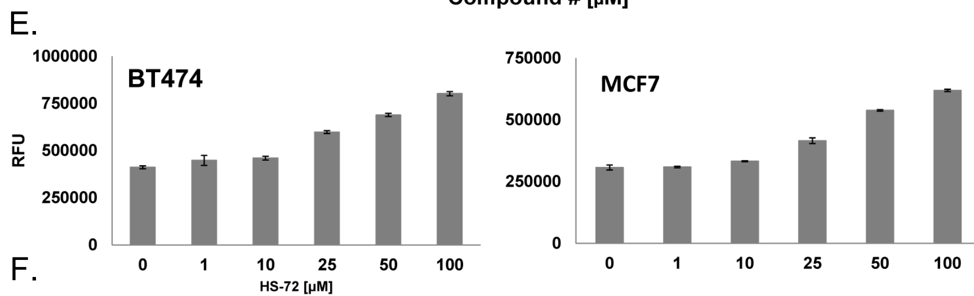
Figure S1, related to Figure 2. Overview of FLECS protocol. (A) ATP resin is mixed with crude cell lysate containing GFP-Hsp70i. (B) Washed beads are distributed into 96-well filter plates. (C) Drug candidates, ATP for a positive control, or buffer as a negative control, were also added to each well. (D) Eluates were separated into a catch plate by centrifugation. (E) The fluorescence of each eluate was measured on a Victor X2 plate reader (Perkin Elmer, Waltham, MA), and a fluorescence histogram was generated. All wells containing >2.5 fluorescence counts above background were considered to contain potential hits.

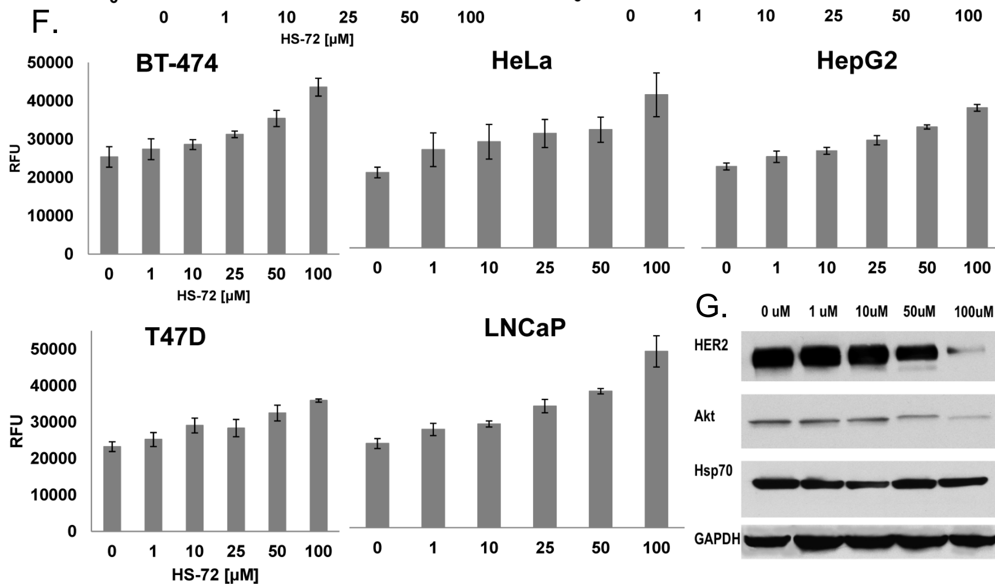
Figure S2




D.
HeLa
RFU
80000
60000
40000
20000
0
Untreated
DMSO
1 (10μM)
1 (50μM)
1 (100M)
3 (10μM)
3 (50μM)
3 (100μM)
4 (10μM)
4 (50μM)
4 (100μM)
9 (10μM)
9 (50μM)
9 (100μM)
13 (10μM)
13 (50μM)
13 (100μM)
14 (10μM)
14 (50μM)
14 (100μM)
Compound # [μM]


BT474
RFU
50000
40000
30000
20000
10000
0
Untreated
DMSO
1 (10μM)
1 (50μM)
1 (100μM)
3 (10μM)
3 (50μM)
3 (100μM)
4 (10μM)
4 (50μM)
4 (100μM)
9 (10μM)
9 (50μM)
9 (100μM)
13 (10μM)
13 (50μM)
13 (100μM)
14 (10μM)
14 (50μM)
14 (100μM)
Compound # [μM]


E.
BT474
RFU
1000000
750000
500000
250000
0
0 1 10 25 50 100
HS-72 [μM]
MCF7
750000
500000
250000
0
0 1 10 25 50 100


F.
BT-474
RFU
50000
40000
30000
20000
10000
0
0 1 10 25 50 100
HS-72 [μM]
HeLa
0 1 10 25 50 100
HepG2
0 1 10 25 50 100
T47D
RFU
50000
40000
30000
20000
10000
0
0 1 10 25 50 100
HS-72 [μM]
LNCaP
0 1 10 25 50 100

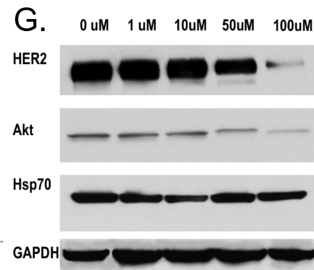

G.
0 uM
1 uM
10uM
50uM
100uM
HER2
Akt
Hsp70
GAPDH

Figure S2, related to Figure 2. (A) Western blotting, confirmed presence of Hsp70i in 60 hits. (Final lead HS-72 is highlighted by box). (B) The 60 hits were tested for ability to elute endogenous Hsp70i from pig bladder tissue bound to the ATP resin. Of the 60 hits tested, 22 hits were confirmed by Western blot. (C) Structures of the 22 hits. (D-F) HS-72 shows cell permeability and hallmarks of Hsp70 inhibition through caspase activation and substrate degradation assays. (D) HeLa or BT474 cells were treated with the 22 hits for 24 hours and caspase activation, a hallmark of Hsp70 inhibition in cancer cells, was measured using the Amplite Fluorimetric Caspase 3/7 assay kit, with fluorescence correlating with caspase activation. Only HS-72 (designated as compound 4) activates caspase-3/7 in HeLa and BT474 cells in a dose dependent manner. (E) HS-72 induces caspase activation in BT474 and MCF7 cells at 6 hours in a dose dependent manner. (F) HS-72 induces caspase activation in BT474, HeLa, HepG2, T47D, and LNCaP cells at 24 hours in a dose dependent manner. (G) HS-72 shows a second hallmark of Hsp70 inhibition by inducing degradation of Hsp70 substrate proteins, HER2 and Akt, in a dose dependent manner in BT474 cells. The levels of Hsp70 remain unchanged, indicating HS-72 is acting to inhibit Hsp70 activity. GAPDH serves as the loading control.
(D-F) Mean ± SEM. RFU, Relative Fluorescence Units.

Figure S3

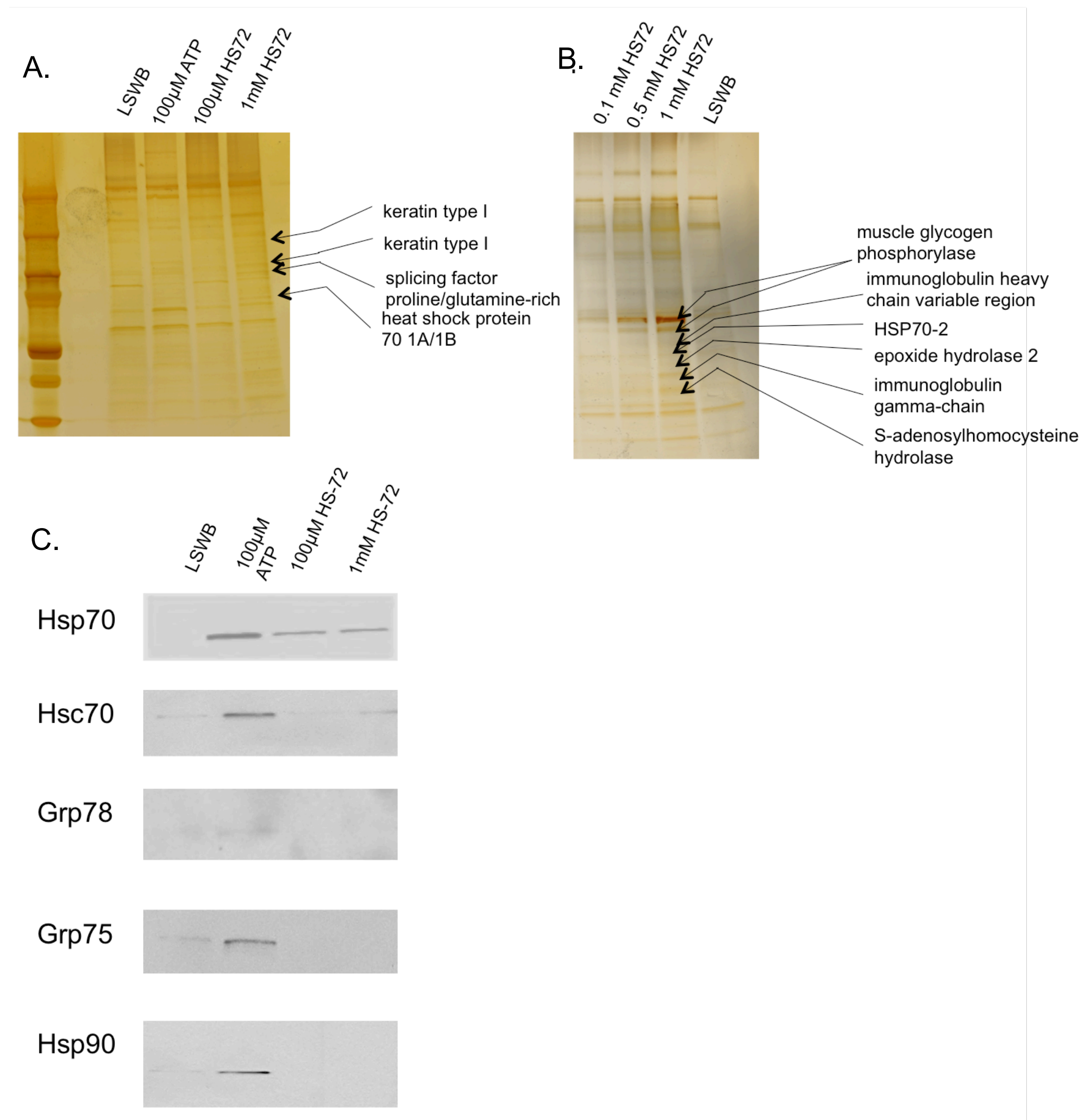


Figure S3, related to Figure 3. (A) HS-72 elutes Hsp70i from HEK 293T cell lysate bound to the ATP resin. (B) HS-72 elutes Hsp70i from pig bladder lysate bound to the ATP resin. (C) HEK 293T cell lysate was eluted with buffer, ATP, or HS-72 from the ATP resin as described in the FLECS assay and subjected to analysis by Western blot. All the Hsp70 family members and Hsp90 are eluted with ATP, while only Hsp70i was eluted with HS-72, highlighting the selectivity of HS-72 for Hsp70i.

Figure S4

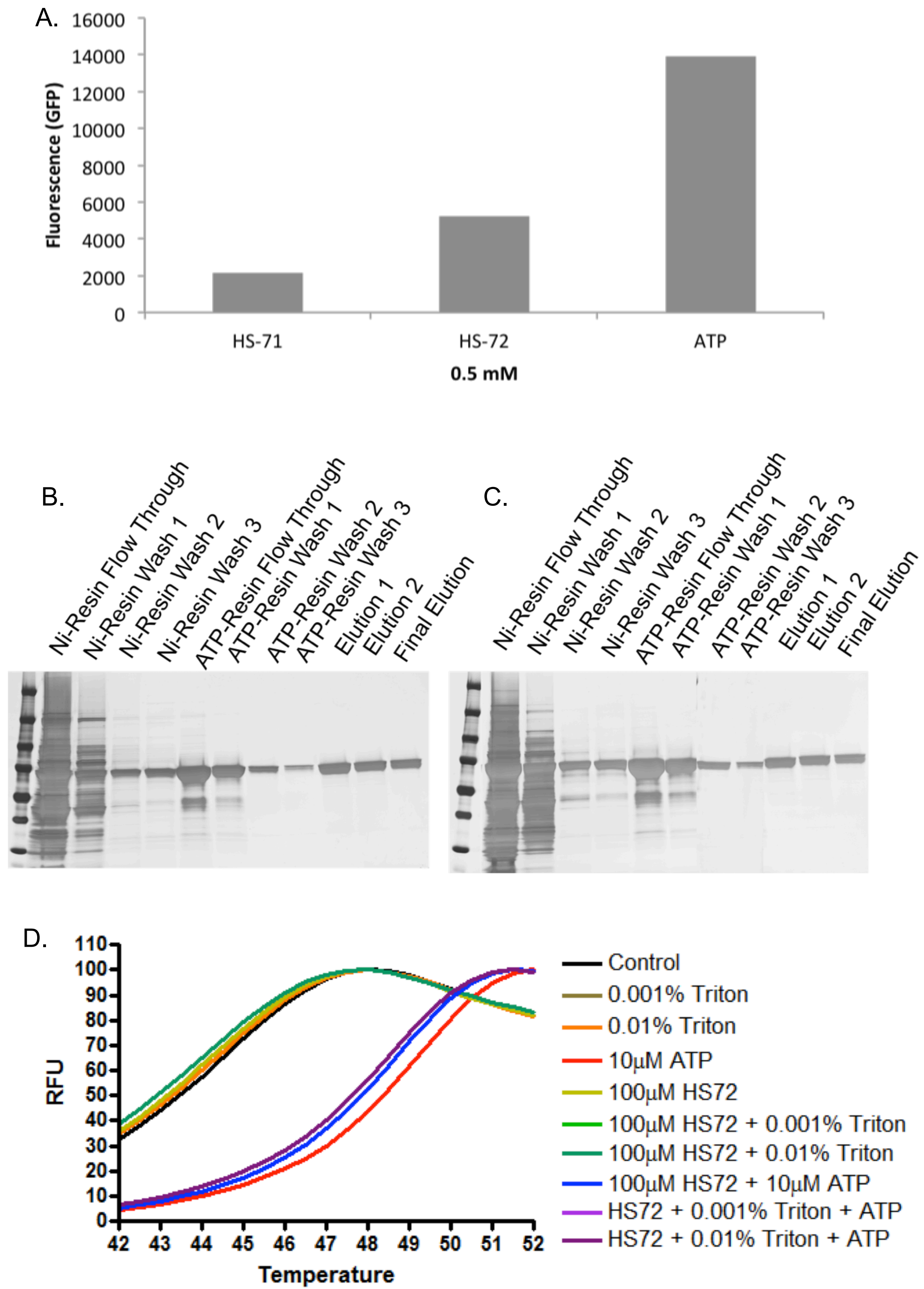
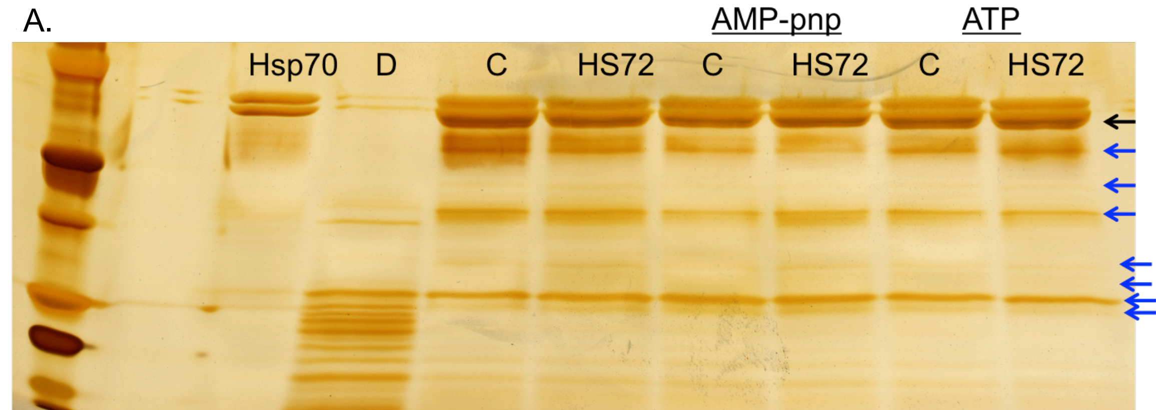


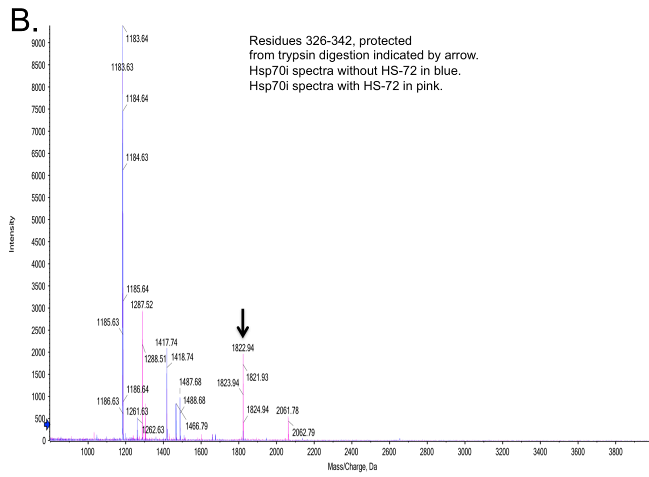
Figure S4, related to Figure 4. (A) HS-72, the S enantiomer, was more effective at eluting GFP-Hsp70 from the ATP resin than the R enantiomer, HS-71. (B-C) Purification of (B) Hsp70i and (C) Hsc70, which was used for Thermofluor and proteolysis studies, shown by silver stain with the final product in the “final elution” sample. (D) The observed decrease in the T_m of Hsp70 by HS-72 is not due to aggregation of the protein as shown using the thermofluor assay in the presence and absence of detergent. There is no difference in the T_m of Hsp70 when comparing 100μM HS72 alone vs. 100μM HS72 + 0.001% Triton or 100μM HS72 + 0.01% Triton. Furthermore, the decrease in T_m in the presence of ATP is observed in the presence of 0.001% Triton and 0.01% Triton.

Figure S5

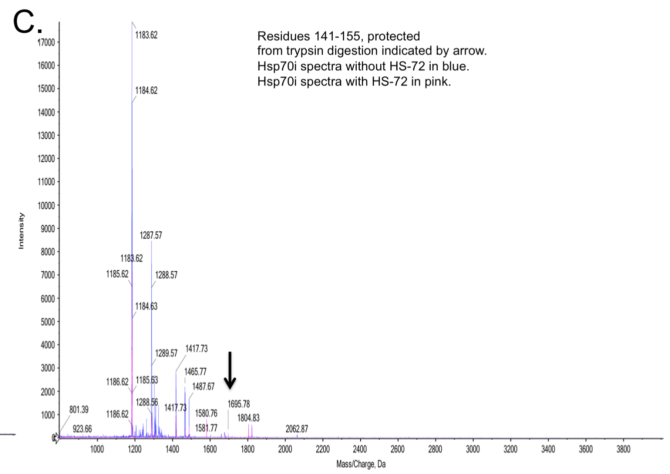
A.



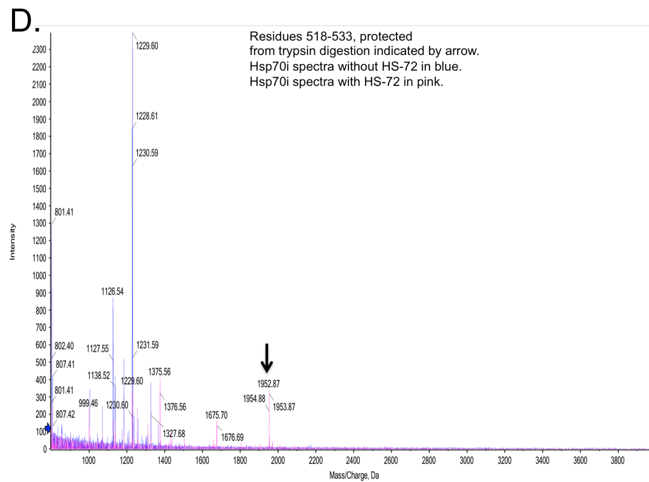
B.



C.



D.



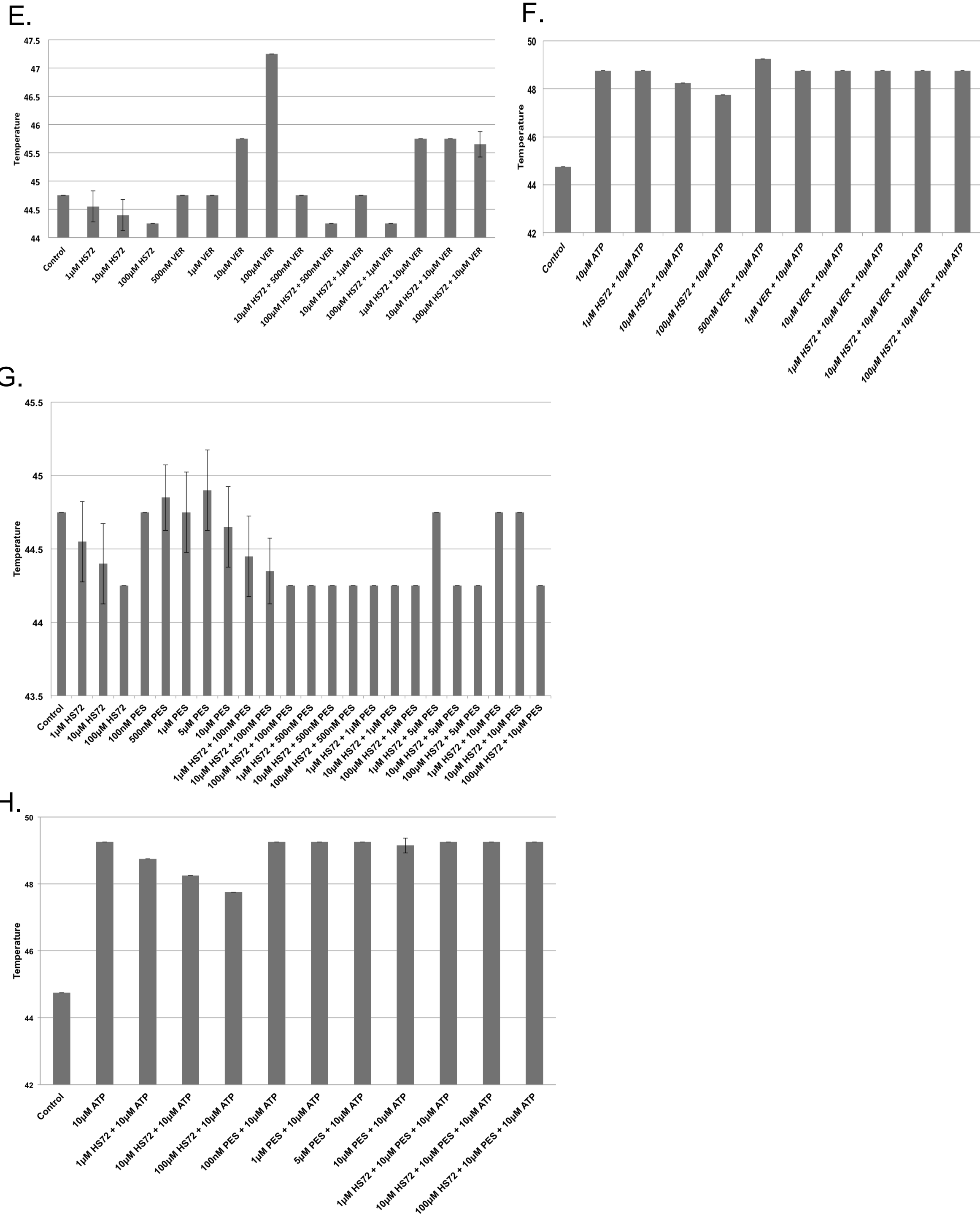
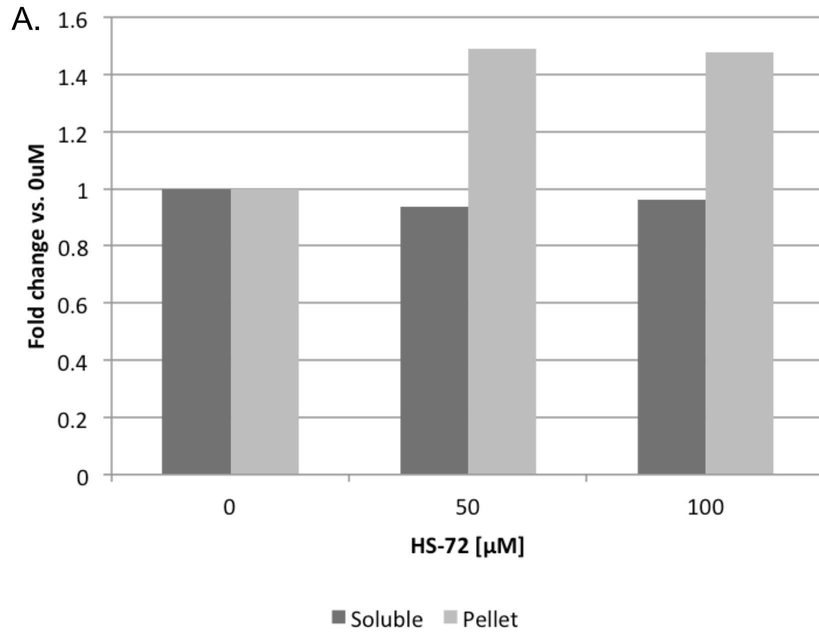


Figure S5, related to Figure 5. (Fig.S5A) Native proteinase K digestion of Hsp70i ± HS-72 at 15 minutes reveals a differential digestion pattern. Reactions were incubated with DMSO (gel lane C) or HS-72 (gel lanes HS72) and the indicated nucleotide (labeled above lanes). A sample of Hsp70 was denatured (gel lane D) by heating in SDS prior to addition of Proteinase K. Undigested Hsp70 (gel lane Hsp70) was included for comparison. Samples were separated by SDS-PAGE and visualized by silver stain. Full length Hsp70i indicated with black arrow, Hsp70i with residual His tag from purification indicated with red arrow, and fragments resulting from proteolysis indicated with blue

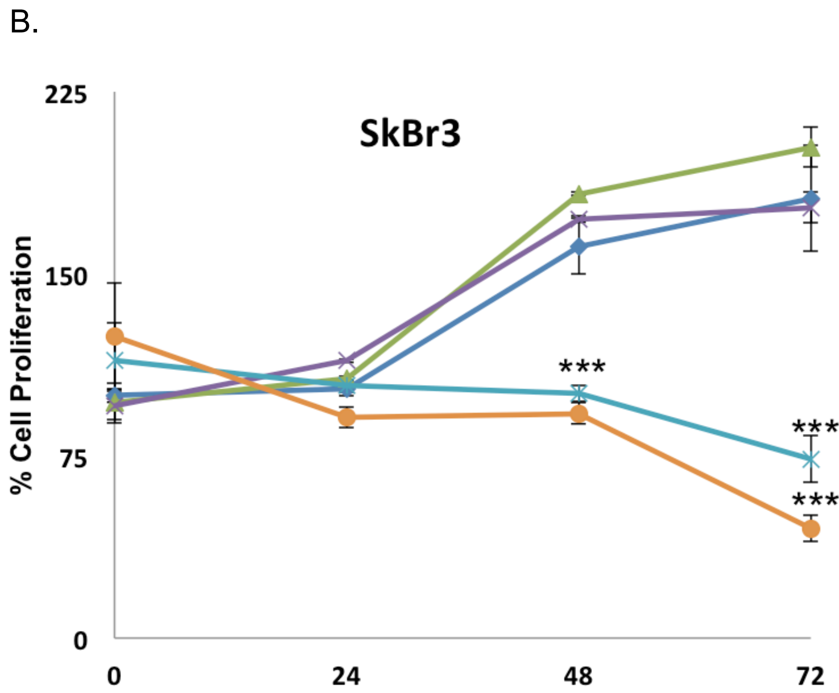
arrows. (Fig.S5B-D). Mass spectra of residues protected from trypsin digestion in the presence of HS-72, which were identified in partial proteolysis analysis. (Fig.S5B) residues 326-342, (Fig.S5C) residues 141-155, and (Fig.S5D) residues 518-533. Hsp70i without HS-72 spectra in blue and Hsp70i with HS-72 spectra in pink. The arrows highlight the indicated residues, which show the differences in spectra. (Fig.S5E-H) HS-72 does not bind Hsp70 in the same site as VER-15508 (VER) or pifithrin-μ (PES) due to no additive or synergistic effects on the T_m of Hsp70i in Thermofluor. HS-72 in combination with VER shows no synergistic or additive interactions between the molecules when tested using Thermofluor in the absence (Fig.S5E) or presence (Fig.S5F) of ATP. HS-72 in combination with PES shows no synergistic or additive interactions between the molecules when tested using Thermofluor in the absence (Fig.S5G) or presence (Fig.S5H) of ATP. (Fig.S5E-H) Mean ± SEM.

Figure S6

A.



B.



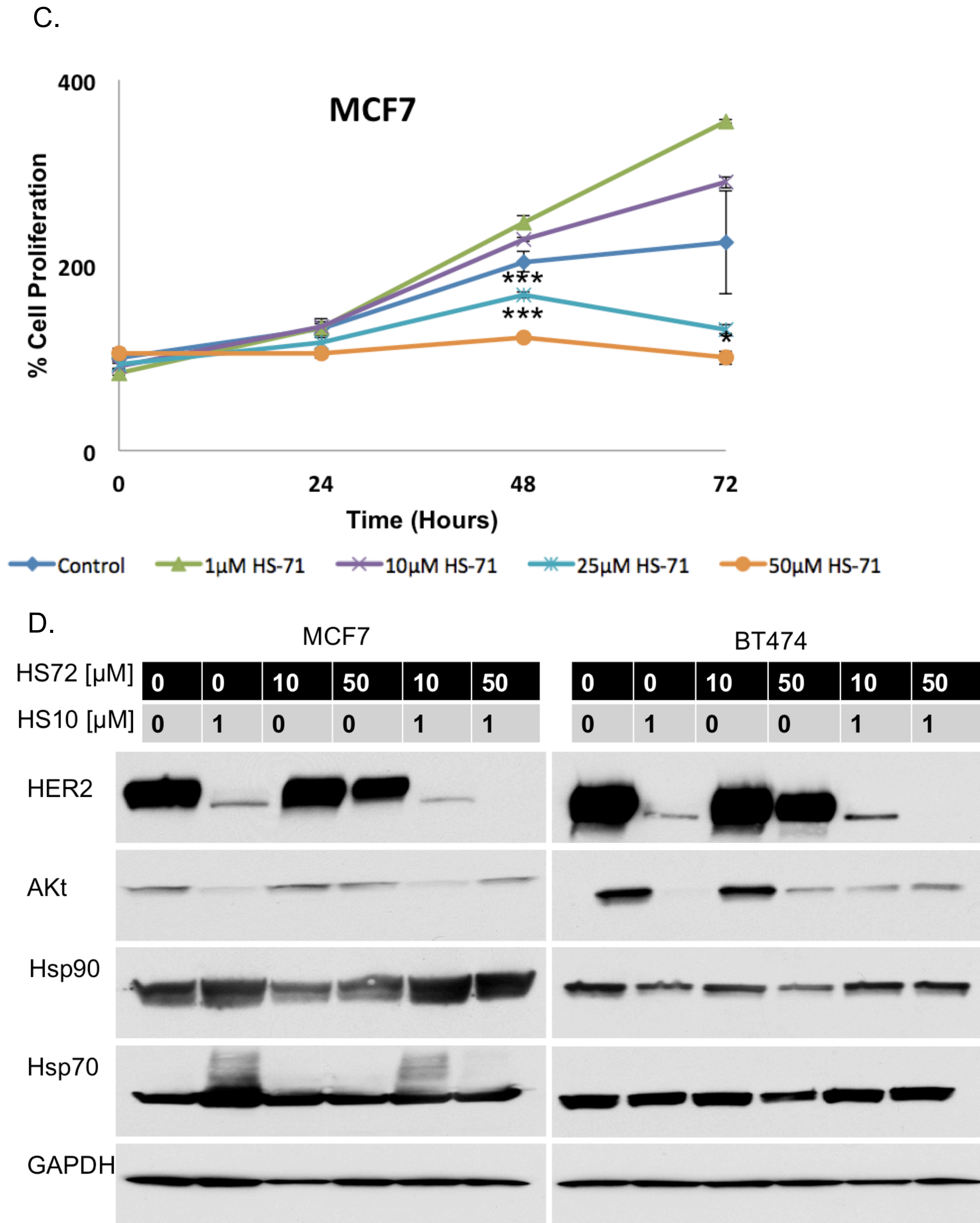
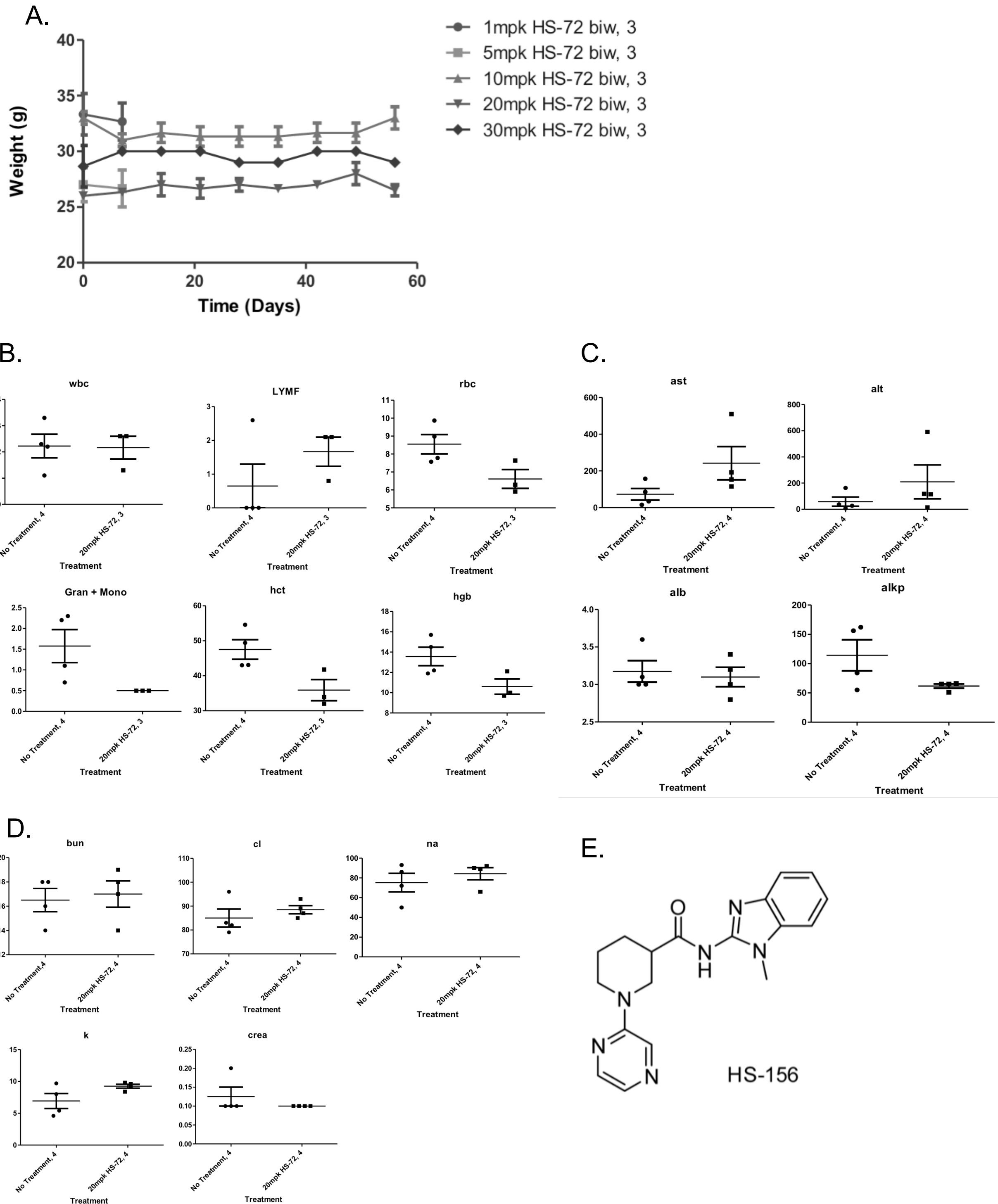
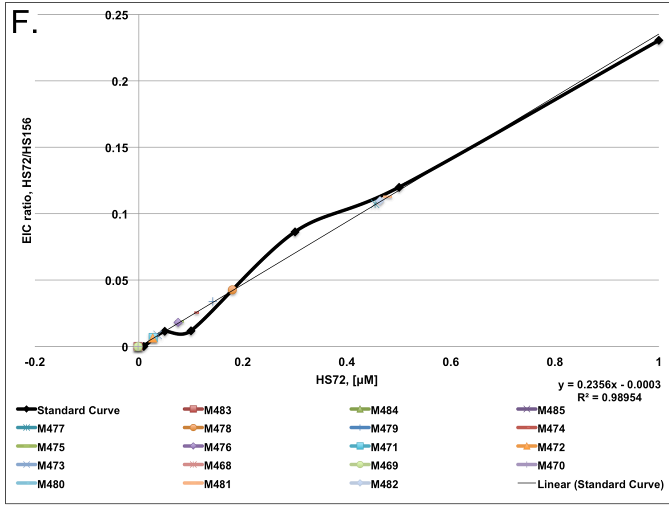


Figure S6, related to Figure 6. (A) HS-72 induces the formation of HttQ-GFP aggregates in a PC12 cell culture model of Huntington's disease. Quantification of bands from the Western blot shown in Figure 6A, illustrating a 50% increase of HttQ-GFP in the insoluble pellet associated fraction in the presence of HS-72. (B-C) HS-71 minimally inhibits cell proliferation and has no effect on proliferation at 24 hours. (B) In SkBr3 cells there is an inhibition in proliferation at 48 and 72 hours at 25µM and 50µM. (C) In MCF7

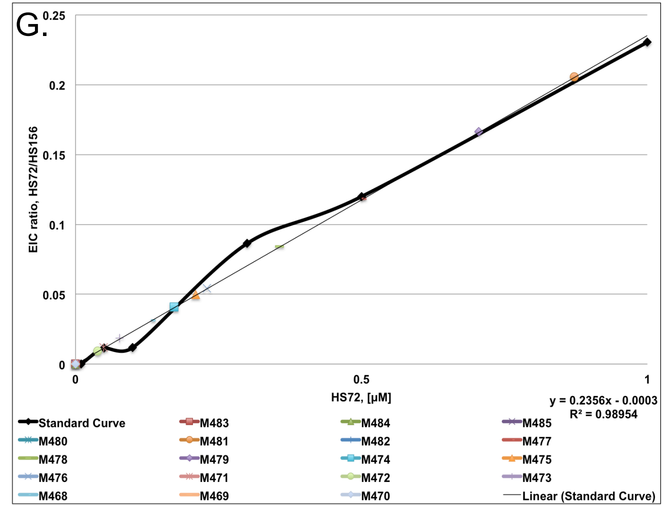
cells there is inhibition at 48 hours at 25μM and 50μM, while at 72 hours significant inhibition is proliferation is observed in 50μM alone. Each cell line was treated with HS-71 at 0 hours and HS-71 treatment was maintained for the duration of the assay. (Mean ± SEM. *, $p<0.05$. ***, $p<0.001$). (D) Combination treatment with HS72 and HS10 induces degradation of Her2 and Akt more efficiently than either HS72 or HS10 alone in MCF7 and BT474 cells. The cells were incubated for 24 hours with the indicated compound, upon which time the cells were harvested and processed for analysis by Western blot for Her2, Akt, Hsp70, Hsp90, or GAPDH. GAPDH serves as the loading control.

Figure S7

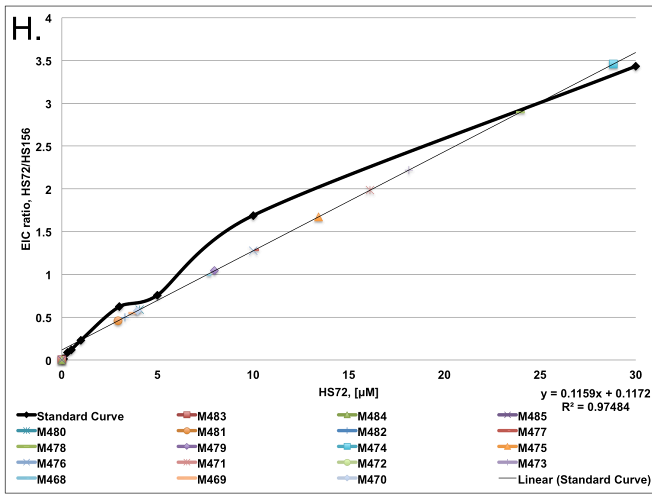




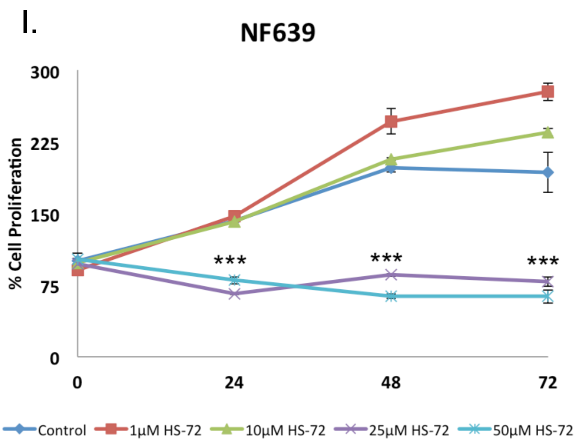
F.
EIC ratio, HS72/HS156
HS72, [µM]
y = 0.2356x - 0.0003
R² = 0.98954
Standard Curve
M483
M484
M485
M477
M478
M479
M474
M475
M476
M471
M472
M473
M468
M469
M470
M480
M481
M482
Linear (Standard Curve)



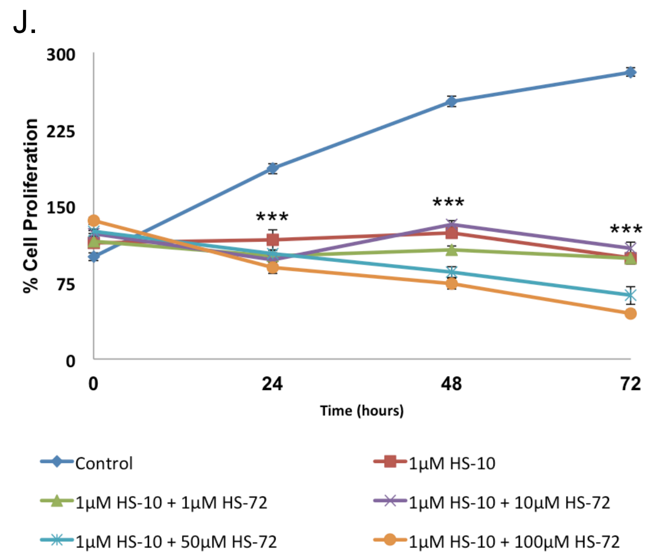
G.
EIC ratio, HS72/HS156
HS72, [µM]
y = 0.2356x - 0.0003
R² = 0.98954
Standard Curve
M483
M484
M485
M480
M481
M482
M477
M478
M479
M474
M475
M476
M471
M472
M473
M468
M469
M470
Linear (Standard Curve)



H.
EIC ratio, HS72/HS156
HS72, [µM]
y = 0.1159x + 0.1172
R² = 0.97484
Standard Curve
M483
M484
M485
M480
M481
M482
M477
M478
M479
M474
M475
M476
M471
M472
M473
M468
M469
M470
Linear (Standard Curve)



I.
NF639
% Cell Proliferation
***
Control
1µM HS-72
10µM HS-72
25µM HS-72
50µM HS-72

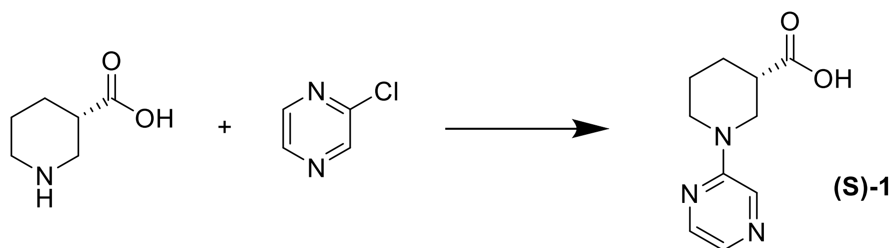


J.
% Cell Proliferation
***
Time (hours)
Control
1µM HS-10
1µM HS-10 + 1µM HS-72
1µM HS-10 + 10µM HS-72
1µM HS-10 + 50µM HS-72
1µM HS-10 + 100µM HS-72

Figure S7, relate to Figure 7. (A) MTD shows that HS72 is well tolerated by wild-type mice and is not toxic at any of the doses tested. Mice were treated I.P. with the indicated doses of HS-72 BiW for 60 days. The 1mpk and 5mpk doses were abandoned after no immediate toxicity at the higher doses. (B) Animals were treated I.P. with 20mpk HS-72 on day 1 and 4 with blood taken for analysis on day 5. Complete blood count (CBC) analysis following HS-72 treatment shows no adverse effect of HS-72. wbc – White blood cell count. LYMF – lymphocytes. rbc – red blood cell count. Gran + Mono – Granuloctye + Monocyte count. hct – hematocrit. hgb – hemoglobin. (C) Liver test following HS-72 treatment showing no adverse effects. Animals were treated as described in S7B. ast – aspartate transaminase. alt – alanine transaminase. alb – albumin. alkp – alkaline phosphatase. (D) Kidney test following HS-72 treatment showing no adverse effects. Animals were treated as described in S7B. bun – blood urea nitrogen. cl – chloride. na – sodium. k – potassium. crea – creatinine. (A-D) Mean ± SEM. (E) Structure of HS-156, which is a close structural analogue of HS-72 and was used as the internal standard for the PK study of plasma, liver, and kidney. (F-H) Plasma, Kidney, and Liver samples plotted on standard curve. Ratio of area under the curve from EIC of HS-72 compared to HS-156 was plotted on the standard curve, which was used to determine concentration of HS-72 in plasma samples. Concentration in solution for all samples adjusted for a 1:4 dilution factor that was used when processing the samples. Final concentration of HS-72 in plasma was calculated per mL of plasma. Final concentration of HS-72 in the kidney and liver was calculated per gram of tissue using the weight of each tissue measured before sample processing. (F) Plasma samples plotted on standard curve, which was used to calculate concentration of HS-72 in each sample. (G) Kidney samples plotted on standard curve, which was used to calculate concentration of HS-72 in each sample. (H) Liver samples plotted on standard curve, which was used to calculate concentration of HS-72 in each sample. (I) HS-72 inhibits proliferation of a MMTV-neu derived cell line, NF639, at 25μM and 50μM at 24, 48, and 72 hours. (J) Combination treatment of HS-72 and HS-10 shows synergistic inhibition of NF639 cell proliferation. NF639 cells treated with indicated compound at 0 hours and treatment was maintained for the duration of the assay. (Mean ± SEM. ***, $p<0.001$).

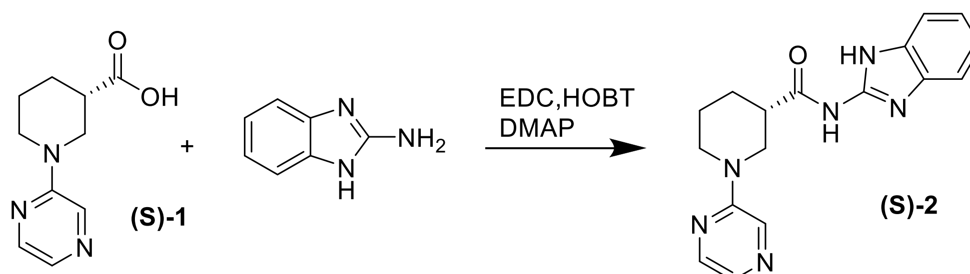
Supplemental Experimental Procedures

Synthesis of HS-72 ((S)-3)

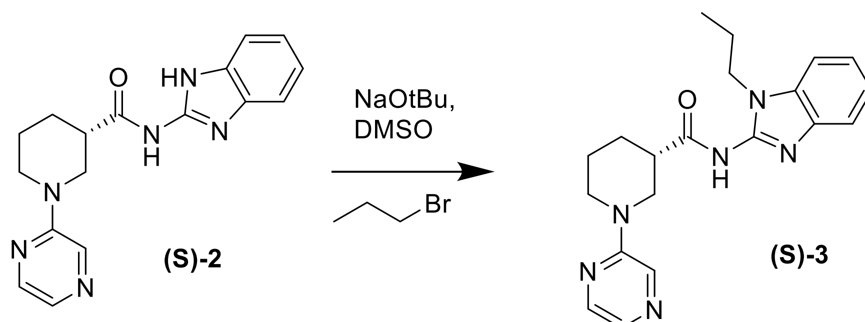


(S)-1-(pyrazin-2-yl)piperidine-3-carboxylic acid **(S)-1**. (S)-(+)-3-Piperidinecarboxylic acid (250 mg, 1.94 mmol) and chloropyrazine (441 mg, 3.87 mmol) were heated together with Hunig's base (500 mg, 3.87 mmol) and ethanol (300 μL) at 120 ºC for 16 h. TLC (4/1/1 : nBuOH/AcOH/H_2O) showed a new product and only a trace of starting material. The reaction mixture was concentrated, dissolved in DMSO and purified by prep HPLC (5 to

100% methanol w/0.2% formic acid, 20 mL/m, Agilent C-18, 21.1 x 25 cm) to give product **(S)-1** (291 mg, 72%) as a white powder. MS (ESI) $[M+H]^+$ m/z = 208.0.



(S)-N-(1H-benzo[d]imidazol-2-yl)-1-(pyrazin-2-yl)piperidine-3-carboxamide **(S)-2**. Compound **(S)-1** (147 mg, 709 mmol) and 2-aminobenzimidazole (189 mg, 1.42 mmol) were mixed with EDC (204 mg, 1.06 mmol), HOBT (96 mg, 0.71 mmol) and DMAP (9 mg, 71 mmol) and Hunig's base (183 mg, 247 mL, 1.4 mmol) and dissolved in DMF (2 mL). TLC (9/1 : CH_2Cl_2/MeOH) showed the slow formation of product and loss of starting material. After 2 h, the reaction mixture was concentrated to remove DMF and chromatographed (gradient CH_2Cl_2 to 9/1 : CH_2Cl_2/MeOH). The product was triturated overnight in ethyl acetate/ hexanes to give **(S)-2** (77.8 mg, 34%) as a white solid. MS (ESI) $[M+H]^+$ m/z = 323.2.



(S)-N-(1-propyl-1H-benzo[d]imidazol-2-yl)-1-(pyrazin-2-yl)piperidine-3-carboxamide **(S)-3**. Amide **(S)-2** (40 mg, 124 umol) was dissolved in DMSO (300 ul) and treated with sodium t-butoxide (124 uL of 2M solution) followed by 1-bromopropane (18.3 mg, 149 µmol) and stirred at RT. After 1 h, TLC (9/1 : CH_2Cl_2/MeOH) showed a new product and a little starting material. The sample was purified by prep HPLC (5 to 100% methanol w/0.2% formic acid, 20 mL/m, Agilent C-18, 21.1 x 25 cm) and recrystallized from ethyl acetate/heptanes to give **(S)-3** (24 mg, 53%) as a white powder. **(S)-3** was identical to commercial racemic **3** by TLC and LC/MS. MS (ESI) $[M+H]^+$ m/z = 365.3.

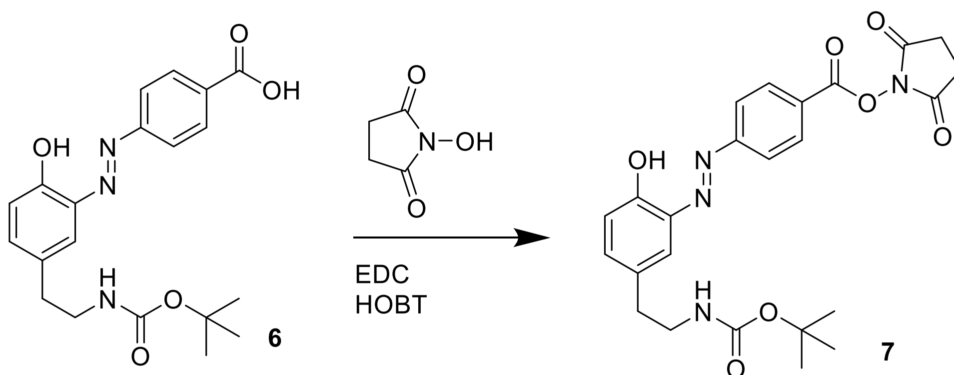
HS-71, (R)-N-(1-propyl-1H-benzo[d]imidazol-2-yl)-1-(pyrazin-2-yl)piperidine-3-carboxamide, **(R)-3**, was prepared in an analogous manner from (R)-(-)-3-piperidinecarboxylic acid.

Synthesis of HS-72 affinity resin ((S)-9).

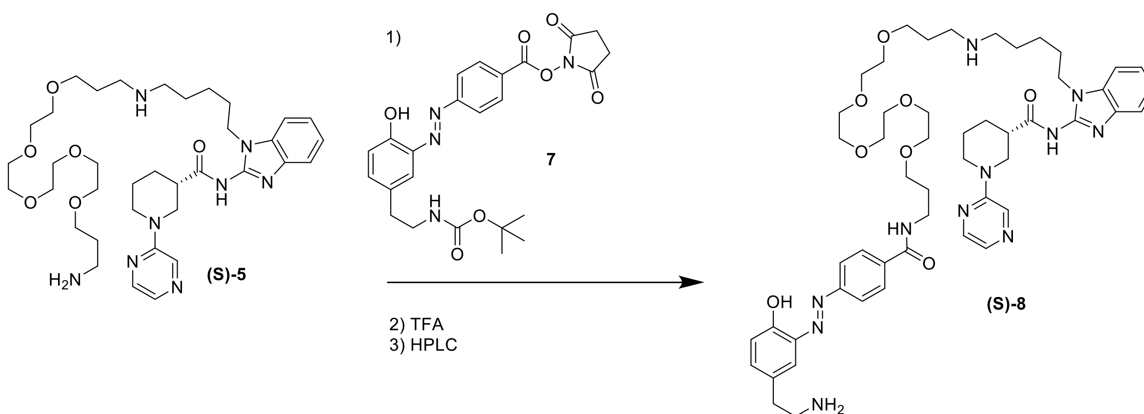
(S)-N-(1-(5-bromopentyl)-1H-benzo[d]imidazol-2-yl)-1-(pyrazin-2-yl)piperidine-3-carboxamide **(S)-4.** Amide **(S)-2** (20 mg, 62 mmol) was dissolved in DMSO (300 ml) and treated with sodium t-butoxide (62 mL of 2 M solution) followed by 1,5-dibromopentane (17 mg, 74 mmol) and stirred RT. After about 1 h, TLC (9/1 : CH_2Cl_2/MeOH) and LC/MS showed a new product (m/z = 471.6). The product was purified by prep HPLC (5 to 100% methanol w/0.2% formic acid, 20 mL/m, Agilent C-18, 21.1 x 25 cm) to give **(S)-4** as a yellow solid. The product was then used for the next reaction.

(S)-N-(1-(1-amino-4,7,10,13,16-pentaoxa-20-azapentacosan-25-yl)-1H-benzo[d]imidazol-2-yl)-1-(pyrazin-2-yl)piperidine-3-carboxamide **(S)-5.** Bromide **(S)-4** (29 mg, 62 mmol) was dissolved in ethanol (2 mL) and treated with 1,19-diamino-4,7,10,13,16-pentaoxanonadecane (60 mg. 195 mmol) followed by DMSO (100mL) and CH_2Cl_2 (1 mL) and stirred at RT for 3 days. The reaction mixture was concentrated and injected onto the prep HPLC (5 to 100% methanol, 20 mL/m, Agilent C-18, 21.1 x 25 cm) to give purified **(S)-5** (12.9 mg, 30% from **(S)-2**) as a glass. LC/MS gives m/z = 669.4 $[M + H]^+$.

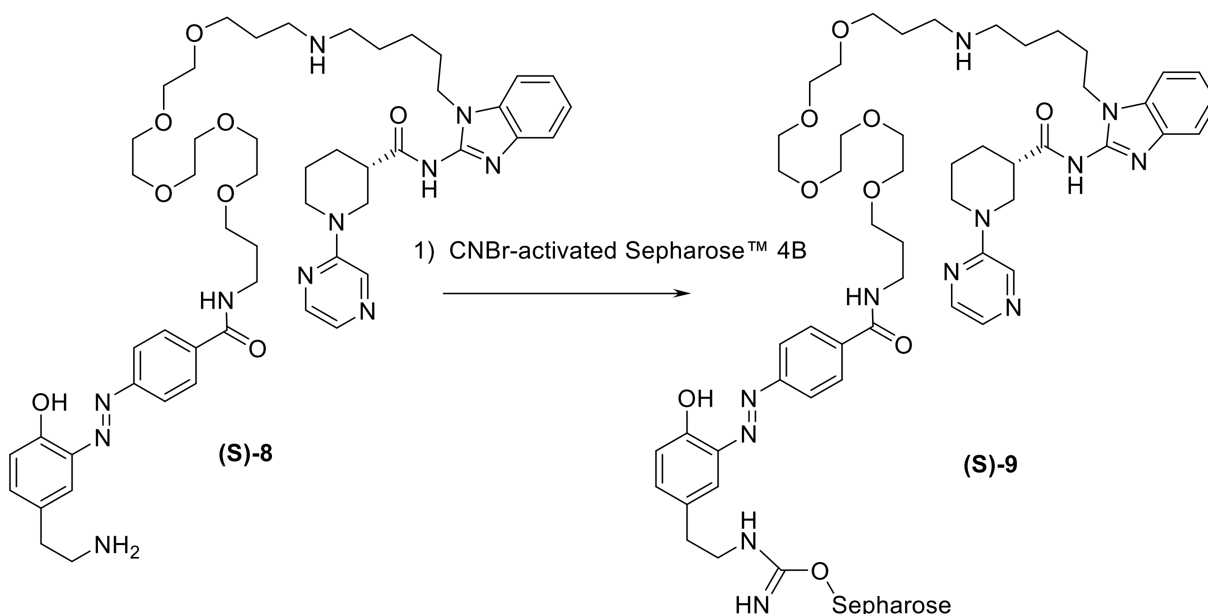
Cleavable linker activation



Activated cleavable linker **7.** The cleavable linker acid (Hughes et al., 2012) **6** (541 mg, 1.4 mmol), EDC (540 mg, 2.8 mmol) and N-hydroxysuccinimide (243 mg, 2.1 mmol) and a chip of DMAP were slurried in dichloromethane (10 mL) and DMF (1.5 mL). After 16 hour, TLC (70% ethyl acetate in hexanes) showed complete reaction. The reaction mixture was added to a column and chromatographed (silica gel, 18 x 3.5 cm, CH_2Cl_2 (200 mL), 25% EtOAc in CH_2Cl_2 (600 mL). The active fractions were combined and concentrated then triturated with hexanes ethyl acetate and filtered off to give the **7** (353 mg, 52%) as an orange powder.



Amine **(S)-8**. Amine **(S)-5** (12.9 mg, 18.5 mmol) was dissolved 9/1: DCM/MeOH (1 mL) and treated with solid activated cleavable linker **7** (25 mg, 52 mmol), followed by Hunig's base (10 mL). After stirring overnight, TLC showed mostly one product and LC/MS showed a big peak in the TIC with the right mass. UV showed nothing at 254 nm as usual. The sample was concentrated to an oil and chromatographed on silica gel eluting with a CH_2Cl_2 to 4/0.9/0.1 : CH2Cl2/MeOH/NH3 gradient. The product was concentrated to give an orange glass. The sample was then dissolved in methylene chloride (1 mL) and TFA (1 mL). After 1 h, TLC (4/0.9/0.1 : CH2Cl2/MeOH/NH3) showed a new product. The mixture was concentrated, diluted with ethanol and concentrated again and then purified by HPLC (5 to 100% methanol, 20 mL/m, Agilent C-18, 21.1 x 25 cm) to give amine **(S)-8** (5.5 mg, 31% overall) as an orange glass. LC/MS showed a single peak with m/z = 966.6 $[M + H]^+$.



Buffers and solutions

Swelling solution 1 mM HCl
Coupling buffer 0.1 M $NaHCO_3$, 0.5 M NaCl, pH = 8.3
Capping solution 1 M ethanolamine
Low buffer 0.1 M AcOH/NaAcOH, 0.5 M NaCl pH = 4
High Buffer 0.1 M TRIS-HCl, 0.5 M NaCl pH = 8
Storage Buffer 0.1M KH_2PO_4, pH = 7.4 w/ 200 mg NaN3/L

Roughly following GE Healthcare Instructions 71-7086-00 AFA.

Affinity resin **(S)-9**. In a 30 mL column, CNBr-activated Sepharose™ 4B (2 g) was swelled in 1 mM HCl (20 ml) and then washed with 1 mM HCl (400 mL). The resin was washed with coupling buffer (20 mL) and then slurried with coupling buffer (10 mL). The mixture was then treated with amine **(S)-8** (5.5 mg) in ethanol (1 mL) and tumbled at RT for 16 h. The resin was then drained (no color eluted) and washed with coupling buffer (5 x 10 mL), diluted with more coupling buffer (~10 mL) and treated with capping solution (200 mL) and rotated for 2 h. The solution was drained and the resin **(S)-9** washed with 3 rounds of high buffer/low buffer (20 mL ea.) and finally washed with water (20 mL) and transferred in storage buffer (10 mL) to a 40 mL EPA vial and stored at 4°C.

Compounds
VER-15508 and pifithrin-μ (PES) were purchased from Sigma (St. Louis, MO) as powders and resuspended in DMSO.

Cell Lines

HEK-293T (ATCC® ACS-4500™), MCF7 (ATCC® HTB-22™), HeLa (ATCC® CCL-2™), HepG2 (ATCC® HB-8065™), T47D (ATCC® CRL-2865™) and NF639 (ATCC® CRL-3090™) cell lines were obtained from ATCC and are grown in DMEM medium supplemented with 10% FBS. BT474, SkBr3, LNCaP, and RWPE1 cell lines were

obtained from Donald McDonnell (Duke University) and were grown in RPMI-1640 medium supplemented with 10% FBS and non-essential amino acids. MCF-10A cell line was obtained from Donald McDonnell and were grown in DMEM/F12 medium supplemented with 5% horse serum, 0.02% EGF, 0.05% Hydrocortisone, 0.01% Cholera Toxin, 0.1% Insulin, and 1% penicillin/streptomycin. PC12 cell line expressing httQ74-GFP was obtained from Dennis Thiele (Duke University) and were grown in DMEM supplemented with 5% FBS, 10% horse serum, 100ug/ml G418, 75ug/ml Hygromycin B, and 100U/ml penicillin/streptomycin plus supplements (Neef et al., 2010). All cell lines were grown at 37°C in an atmosphere of 5% CO_2.

Western Blotting

SDS-PAGE was carried out using Criterion^{TM} Cell system using pre-casted 4-20% or 4-15% Criterion^{TM} Tris-HCl gels (BioRad, Hercules, CA). For Western blotting gels were run at 200V for 1 hour using the PowerPac basic power supply (BioRad, Hercules, CA). Next, gels were transferred to nitrocellulose for blotting at 100V for 1 hour (Fisher Scientific, Waltham, MA). Nitrocellulose membranes were blocked with 5% dry non-fat milk in phosphate-buffered saline (PBS) with 0.01% Tween-20 for 1 hour at room temperature. Membranes were incubated with primary antibodies (1:1000 dilution) overnight at 4°C. The next day membranes were washed 3X in PBS-Tween, incubated for 1 hour at room temperature with secondary antibodies, and further washed 3X in PBS-Tween. ECL Plus Western blotting reagent (Pierce Biotechnology, Rockford, IL) was used to detect antibodies. GFP, Her2, Akt, Hsp70, Hsc70, Grp78, Grp75, and Hsp90 primary antibodies and all associated secondary antibodies were purchased from Cell Signaling Technology (Danvers, MA).

Protein Purification

Jason Gestwicki (University of California, San Francisco) generously provided plasmids for human HSPA1A, HSPA8, and HSPA1A C306D. Plasmids were transformed in Rosetta competent cells and single colonies were picked from streaked LB/ampicillin/chloramphenicol plates. Cultures were grown at 37°C for 4-6 hours, were cooled to 15°C and expression was induced overnight with 200uM isopropyl 1-thio-β-D-galactopyranoside. Cells were pelleted and resuspended in Ni-lysis buffer (50mM Na2HPO4, 300mM NaCl, 10mM Imidazole, 0.05% Tween-20) supplemented with Complete Mini protease inhibitor tablets (Roche, Mannheim, Germany) and 1M DTT, and then sonicated. The cells were again pelleted and the supernatant was incubated with cOmplete His-Tag purification resin (Roche, Mannheim, Germany) for 5 hours at 4°C. The resin was washed with Ni-wash buffer (50mM Na2HPO4, 300mM NaCl, 20mM Imidazole, 0.05% Tween-20) and eluted with Ni-elution buffer (50mM Na_2HPO_4, 300mM NaCl, 250mM Imidazole, 0.05% Tween-20). The elution was then incubated with tobacco etch virus protease overnight at 4°C. The following day the elution was incubated with the γ-phosphate ATP sepharose resin for 30 minutes at 4°C. Next the resin was washed with ATP-wash buffer (50mMTris-HCl, 60mM $MgCl_2$, 60mM KCl, 10mM Citrate) supplemented with 1mM DTT and then ATP-wash buffer supplemented with 2mM ATP was added to the resin to elute the protein. The final elutions were concentrated in Amicon Ultra-15 Centrifugal Filter Units (EMD Millipore, Billerica, MA) and buffer exchanged into 25mM HEPES, 5mM $MgCl_2$, 10mM KCl (pH 7.5) and stored at -80°C until use.

Endogenous Hsp70 elution

Pig bladder tissue was used for eluting endogenous Hsp70 from the γ-phosphate ATP sepharose resin. Tissue was homogenized in liquid nitrogen and stored at -80°C until further use. Tissue mass was measured (g) and then 2.5 X volume (mL) of tissue lysis buffer (50 mM HEPES, 60 mM MgCl2, 60 mM KCl, 1 mM DTT) was added and homogenized on a laboratory blender. A total of 25 grams of homogenized tissue was typically used for each experiment. Subsequent to lysis, the tissue was centrifuged at 35,000 RPM in a Beckman Type 45 Ti rotor (Brea, CA) for 45 minutes at 4°C, and then filtered over silica wool. The filtered supernatant was then added to the γ-phosphate ATP sepharose resin and washed as previously described in the FLECS screen. Compounds were used to elute Hsp70 and the elutions were then separated by SDS-PAGE and analyzed by Western blot or the gels were visualized by silver stain.

Degradation Assay

The specified cells were seeded overnight and then treated with the indicated concentration of compound for 24 hours. The cells were then harvested and subjected to analysis by Western blot.

Cell Proliferation

Cell proliferation was determined using a Hoechst stain (Sigma, St. Louis, MO) to quantify DNA. 5,000 cells of the designated cell line were plated in 96 well plates and treated the next day, designated as time point 0, with the indicated concentration of the specified compound maintained for the duration of the assay. At the indicated time points the media was removed and plated frozen at -80°C. Double distilled H_2O was then added and the plates were incubated at 37°C for 1 hour. After 1 hour the plates were frozen at -80°C. After freezing the plates were thawed and Hoechst stain was diluted 1:1000 in TNE buffer (10mM Tris, 2M NaCl, 1mM Na_2EDTA). The final fluorescence was measured on the Victor X2 plate reader at 355/460 nm, and cell proliferation was determined with the formula: % Cell Proliferation = 100 x (Sample fluorescence – background fluorescence) / (Control fluorescence – background fluorescence).

Mass Spectrometry

Tryptic peptides were subjected to matrix-assisted laser desorption-ionization mass spectrometry (MALDI-MS) on an ABSCIEX TOF/TOF 5800 mass spectrometer. Positive mode time of flight was used to identify peptides, and individual peptides were sequenced by MS/MS. All sequence and peptide fingerprint data was searched using the UniProt database.

ATPase Assay

Single turnover assays were performed as previously described (Fewell et al., 2004). Briefly, Hsp70 was incubated with ^{32}P-ATP and cold ATP in single turnover buffer (1M KCl, 250mM HEPES, 110mM MgOAc) for 30 minutes on ice. ^{32}P-ATP-Hsp70 complex was purified from a Nick Column-Sephadex G-50 (Amersham, Piscataway, NJ). Glycerol was added and the complexes were stored at -80°C. To determine ATP hydrolysis, a sample was thawed and added to single turnover buffer containing Hlj1 and compound added after 60 seconds. At the specified time points an aliquot of the reaction is

removed, added to stop solution (2M LiCl, 4M formic acid, 36mM ATP) and spotted on a TLC plate. The percentage of ATP hydrolyzed to ADP and P_i was then calculated.

Limited Proteolysis

Limited proteolysis was performed as previously described (Seguin et al., 2012). Briefly, 4μg of purified Hsp70i was incubated with HS-72, DMSO, and/or the indicated nucleotide for 20 minutes on ice. 1.8ng of Proteinase K was added and incubated at 37°C for 5 minutes. The reaction was quenched with 100% TCA and incubated for 10 minutes on ice. Reactions were centrifuged for 10 minutes at 13,000 rpm at 4°C and the supernatants were removed. The pellets were resuspended in TCA sample buffer (80 mM Tris HCl pH 8, 8 mM EDTA, 120 mM DTT, 3.5% SDS, 0.29% glycerol, 0.08% Tris base, 0.01% bromophenol blue), separated by SDS-PAGE, and visualized by silver stain.

HS-72 *in vivo* MTD and blood workup

The 5 cohorts consisted of 3 mice each given HS-72 BiW and administered IP in DMSO at 1, 5, 10, 20 and 30mpk using female FVBs aged to 10 weeks. Body Mass was measured weekly and the mice were monitored for signs of toxicity as per MP1U standard protocol. For blood analysis, 4 mice were injected IP with HS-72 on day 1 and 4, with blood drawn on day 5. 4 mice receiving no treatment were used as controls.

HS-72 PK

Wild-type mice were injected IP with HS-72 and sacrificed 5 minutes, 1 hour, 4 hours, 8 hours, and 24 hours post injection. Untreated animals were included as control and called 0 minutes. Each time point consisted of 3 animals. At the indicated time points liver, kidney, and blood was harvested from each animal. Whole blood was centrifuged and only the plasma was retained. Whole liver and kidneys were frozen and stored at -80°C until processing. Before quantifying HS-72 in the plasma and tissue a standard curve was made using HS-72 and HS-156, a close structural analogue to HS-72 as the internal standard. HS-72 and HS-156 were diluted in water and were further diluted 1:4 in acetonitrile. The resulting solution was then filtered through a 0.2μm PTFE membrane (VWR, Radnor, PA). LCMS analyses were performed on an Agilent 2100 LC system (Santa Clara, CA) connected to an Agilent 6311 Ion Trap LCMS. Samples were injected (15 μL injection volume) onto an Agilent Eclipse Plus C18 column (4.6 mm i.d. × 150 mm, 5 μm particle size) at room temperature. Mobile phases A (0.2% formic acid in water) and B (0.2% formic acid in acetonitrile) were mixed to form a gradient of 5%-100% B over 9 minutes. MS analysis was performed in positive ionization mode (Nebulizer 50 psi, Dry Gas 12.0 L/min, Dry Temperature 350 °C, Capillary 31 nA, Ramp Range 4.5-1.5 kV). The detection mass target was 350 m/z and the scan range was 100-1000 m/z. Data was analyzed on Bruker Data Analysis software for 6300 Series Ion Trap v.4.0 sp2. Extracted ion chromatograms (EIC) were generated for target masses $[M+H]^+$, m/z 337 and $[M+H]^+$, m/z 365 and peak areas were determined using standard integration protocol or manual integration. The total ion chromatogram (TIC), UV chromatogram, and mass spectra (MS) were also obtained for each run. The area under the curve from the EIC for HS-72 $[M+H]^+$, m/z 365 and for HS-156 $[M+H]^+$, m/z 337 was determined. The EIC area ratio for HS-72 compared to HS-156 was calculated and this ratio was used to plot a standard curve based on the known concentrations of HS-72. The plasma samples were processed by diluting 1:4 in acetonitrile, and filtered through a 0.2μm PTFE membrane. 15uL of the solution was run through an Eclipse Plus C18 column and analyzed by an Agilent Ion Trap 6130 LC-MS. The resulting EIC ratios from

HS-72 compared to HS-156 were then determined, which was used to calculate the concentration of HS-72 in the diluted sample. The concentration in solution of HS-72 was adjusted for the 1:4 dilution that occurs during sample preparation. The final concentration of HS-72 in plasma was calculated per mL of plasma. The liver and kidney samples were weighed and homogenized in tissue lysis buffer. The resulting homogenate was then processed and analyzed in the same manner as the plasma samples. The resulting EIC ratios from HS-72 compared to HS-156 were then determined, which was used to calculate the concentration of HS-72 in the diluted sample. The concentration in solution of HS-72 was adjusted for the 1:4 dilution that occurs during sample preparation. Final concentration of HS-72 in the kidney and liver was calculated per gram of tissue using the weight of each tissue measured before sample processing. The raw files for all samples are contained in Supplemental material 2 with the TIC, EIC, UV chromatogram, and MS on the pages titled “Display Report – All Windows Selected Analysis”. The raw values for the EIC area under the curve for HS-72 and HS-156 are located on the pages titled “Compound Mass Spectrum List Report – MS”.

Statistical analysis

All statistical analysis performed using GraphPad Prisim4 (La Jolla, CA). Significance determined as $p < 0.05$. Thermofluor data was analyzed using a one-way ANOVA with a Newman-Keuls posttest, mean ± SEM. All proliferation data was analyzed using a two-way ANOVA with a Bonferroni posttest, mean ± SEM. Tumor growth analysis was analyzed using a two-way ANOVA with a Bonferroni posttest, comparing the HS-72 treated animals and control animals at each time point, mean ± SEM. Tumor growth was also analyzed by linear regression to compare the slope of the lines between the HS-72 treated animals and control animals.

Supplemental References

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