Supporting Information

Caldas-Lopes et al. 10.1073/pnas.0903392106

SI Materials and Methods

Cell Lines. The human TNBC cells MDA-MB-468, MDA-MB-231, and HCC-1806 were obtained from the American Type Culture Collection. Luminal-like breast cancer cells MDA-MB-361, MDA-MB-453, BT-474, SKBr-3, UACC-893, MCF-7, T47D, and ZR-75 were a gift from Dr. Neal Rosen, MSKCC. Cells were cultured routinely in DME-HG or RPMI (SKBR-3 and ZR-75–1) supplemented with 10% FBS, 1% L-glutamine, 1% penicillin, and streptomycin.

Reagents. Hsp90 inhibitors (1) were synthesized as previously reported (2). For in vivo administration, PU-H71 was dissolved in PBS (pH 7.4). Otherwise, all compounds were used as DMSO stocks. The ERK2 (#328006), the PKCα/β (#539654), the Tyk2 (#658390), and the Akt1/2 (#124018) inhibitors were purchased from Calbiochem and used as suggested by the vendor, and at concentrations reported to confer selectivity for their target.

Growth Inhibition Assay. The antiproliferative effects of select Hsp90 inhibitors was evaluated using the CellTiter-Glo Luminescent Cell Viability Assay kit (#G7571; Promega). The amount of ATP is directly proportional to the number of cells present in culture. In consequence, loss of ATP levels in cells may be used as an indicator of cell death, while cell proliferation may be recognized by increased levels of ATP. Briefly, exponentially growing MDA-MB-468, MDA-MB-231, and HCC-1806 cells were seeded into black 96-well microtiter plates (#3650; Corning) and incubated in medium containing either vehicle control (DMSO) or compounds for the indicated time at 37 °C. Plates containing 3 replicate wells per assay condition were seeded at a density of 8×10^3 cells for each cell line in 100 μL medium. After exposure of cells to the Hsp90 inhibitors, plates were equilibrated to room temperature (20–25 °C) for approximately 30 min, and 100 μL CellTiter-Glo reagent were added to each well. Plates were mixed for 2 min on an orbital shaker and then incubated for 15 min to 2 h at room temperature (optimal incubation time was determined for each cell line). The luminescence signal in each well was measured in an Analyst GT microplate reader (Molecular Devices). The percentage cell growth inhibition was calculated by comparing luminescence readings obtained from treated versus control cells, accounting for initial cell population (time 0). The IC_{50} was calculated as the drug concentration that inhibits cell growth by 50%.

Hsp90 Binding Assay. Measurements were performed in black 96-well microtiter plates (#3650; Corning) as previously described (3, 4). In short, cell lysates were prepared by rupturing cellular membranes by freezing at -70 °C and dissolving the cellular extract in HFB [20 mM Hepes (K), pH 7.3, 50 mM KCl, 5 mM $MgCl_2$, 20 mM Na_2MoO_4, 0.01% Nonidet P-40] with added protease and phosphatase inhibitors. Saturation curves were recorded in which fluorescently labeled geldanamycin (Cy3B-GM) (3 nM) was treated with increasing amounts of cellular lysates. The amount of lysate that resulted in polarization (mP) readings corresponding to 90%–99% bound ligand was chosen for the competition study. Here, each 96-well plate contained 3 nM Cy3B-GM, cellular lysate (amounts as determined above and normalized to total Hsp90 as determined by Western blot analysis using Hsp90 purified from HeLa cells as standard (#SPP-770; Stressgen) and tested Hsp90 inhibitor in a final volume of 100 μL. The plate was left for 24 h on a shaker at 4 °C, and the fluorescence polarization (FP) values in mP were recorded. EC_{50} values were determined as the competitor concentrations at which 50% of the Cy3B-GM was displaced. FP measurements were performed on an Analyst GT microplate reader (Molecular Devices).

Western Blotting. Cells were grown to 60–70% confluence and exposed to PU-H71 or DMSO vehicle for the indicated times. Lysates were prepared using 50 mM Tris, pH 7.4, 150 mM NaCl, and 1% Nonidet P-40 lysis buffer. Protein concentrations were determined using the BCA kit (Pierce) according to the manufacturer's instructions. Protein lysates (20–100 μg) were electrophoretically resolved by SDS/PAGE, transferred to nitrocellulose membrane, and probed with the indicated primary antibodies: Anti-Hsp90 from mouse (1:500, SPA-830; Stressgen), anti-Hsp70 from mouse (1:500, SPA-810; Stressgen), anti-Akt from rabbit (1:500, 9272; Cell Signaling), anti-phospho-Akt (Ser 473) from rabbit (1:500, 9271S; Cell Signaling), anti-phospho-PDK-1 from rabbit (1:1,000, 3061; Cell Signaling), anti-Raf-1 from rabbit (1:300, sc-133; Santa Cruz Biotechnology), anti-PARP (p85 fragment) from rabbit (1:250, G7341; Promega), anti-EGFR from mouse (1:2,000, 2234; Cell Signaling), anti-HER3 from rabbit (1:250, sc285; Santa Cruz Biotechnology), anti-Tyk2 from rabbit (1:200, sc169; Santa Cruz Biotechnology), anti-*p*-Tyk2 from goat (1:200, sc11763; Santa Cruz Biotechnology), anti-BcL-xL from rabbit (1:1,000, 2762; Cell Signaling Technology), anti-PKCβ from rabbit (1:500, A00903; GenScript), anti-PKCβ from mouse (1:500, 610127; BD Transduction Laboratories), anti-*p*-PKCβ from rabbit (1:500, A00654; GenScript), anti-*p*-PKCα/β from rabbit (1:500, 9375; Cell Signaling), anti-ERK1/2 from mouse (1:1,000, 9106; Cell Signaling), anti-p90RSK from rabbit (1:1,000, 9347; Cell Signaling), anti-*p*-p90RSK from rabbit (1:1,000, 9341; Cell Signaling), anti-IRAK-1 from rabbit (1:200, sc7883; Santa Cruz Biotechnology), anti-TBK1 from rabbit (1:2,000, 05–856; Upstate Biotechnology), anti-Tab2 from rabbit (1:200, sc20756; Santa Cruz Biotechnology), and anti-CSK from rabbit (1:200, sc-13074; Santa Cruz Biotechnology). Membranes were then incubated with a 1:5,000 dilution of a peroxidase-conjugated corresponding secondary antibody. Equal loading of the protein samples was confirmed by parallel Western blots for β-actin (1:5,000, ab8227–50; Abcam) or anti-PI3K (p85) from rabbit (1:4,000, 06–195; Upstate Biotechnology). Detection was performed using the ECL-Enhanced Chemiluminescence Detection System (Amersham Biosciences) according to the manufacturer's instructions. Blots were visualized by autoradiography.

Densitometry. Gels were scanned in Adobe Photoshop 7.0.1 and quantitative densitometric analysis was performed using Un-Scan-It 5.1.

Flow Cytometric Analyses. ***Propidium Iodide Staining.*** Cells were treated with the indicated concentrations of drugs for 24 or 48 h, and analysis of the intracellular DNA content was performed. Briefly, after incubation for 24 and 48 h, cells were washed with PBS, fixed in cold 70% ethanol (in PBS) for at least 30 min at 4 °C, and washed twice more with cold PBS. Cells were stained by the addition of 50 μL solution of propidium iodide (1 mg/mL) (Sigma-Aldrich) and 20 μL ribonuclease-A (10 μg/mL) (Sigma-Aldrich), and DNA content was analyzed by flow cytometry in a FACScan (BD Biosciences). Data were analyzed using FlowJo software, and the distribution of cells in different phases of the

cell cycle was determined as a ratio of the fluorescent area of the appropriate peaks to the total fluorescent area.

Mitotic cell staining. MDA-MB-468 cells treated for 24 h with 0.5 and 1.0 μM PU-H71 were fixed in cold 70% ethanol as described above, then incubated with an anti-phosphohistone H3 (Ser-10) from rabbit (1:200, 06570; Upstate Biotechnology) followed by an anti-rabbit IgG-FITC (1:200; Jackson Immunologicals). Cells were stained with propidium iodide and analyzed by flow cytometry in a FACScan.

Apoptosis Assays. Induction of apoptosis was evaluated through quantification of several variables by flow cytometry upon exposure to 1 μM PU-H71 for 48 h.

Annexin V binding. Binding of phosphatidyl-serine residues to Annexin V-FITC is indicative of early apoptotic changes in cell membrane composition. Following exposure to PU-H71, cells were trypsinized, washed with PBS, and resuspended in Annexin-V Binding Buffer in the presence of Annexin-V FITC (BioSource International). After a 15-min incubation at room temperature in the dark, cells were analyzed by flow cytometry. Apoptotic cells showed FITC staining above background, DMSO-exposed cells.

Mitochondrial permeability assay. Loss of mitochondrial membrane potential follows translocation and pore formation by BH3-domain containing proteins, and ensures mitochondrial collapse and release of pro-apoptotic factors, such as cytochrome C. Loss of mitochondrial membrane potential was detected using the Mitochondrial Permeability kit according to instructions from the manufacturer (Biomol). Briefly, cells were incubated in mitochondrial dye at 37 °C for 10–15 min in presence of CO_2, then centrifuged, and washed in warm assay buffer 3 times before analysis by flow cytometry. The apoptotic cell population presented a lower red fluorescent intensity than the negative control population.

TUNEL assay. The DeadEnd Fluorometric TUNEL system (Promega) was used to measure nuclear DNA fragmentation, a later indicator of apoptosis. Cells were fixed in Cytofix/Cytoperm followed by fixed in 70% ethanol and stored at -20 °C for at least 4 h. Cells were stained in 50 μL incubation buffer containing equilibration buffer, recombinant terminal deoxynucleotidyl transferase, and fluorescein-12-dUTP, in a water bath for 60 min at 37 °C, protecting from direct light exposure. The reaction was terminated by adding 1 mL 20 mM EDTA. Cells were centrifuged and permebilized in 0.1% Triton X-100 solution in PBS containing 5 mg/mL BSA. Lastly, cells were centrifuged and reuspended in 0.5 mL propidium iodide solution (freshly diluted to 5 μg/mL in PBS) containing 250 μg DNase-free RNase A and analyzed by flow cytometry. Fluorescein-12-dUTP incorporation indicated the presence of DNA-ends, whereas propidium iodide stained both apoptotic and nonapoptotic cells red.

Propidium iodide staining. Presence of cells with fragmented DNA can also be detected as cells with less than 2 N DNA content (subG1). For DNA content analysis, cells were washed in ice-cold PBS and fixed in 70% ethanol. Fixed cells were centrifuged at 1,200 rpm for 5 min and stained by incubation in PBS containing 50 μg/mL propidium iodide (Sigma-Aldrich) and 50 μg/mL DNase-free RNase (Roche). Data for all assays were collected with Cell Quest Pro software (Becton Dickinson) from no fewer than 10,000 cells and analyzed with FlowJo.

YO-PRO-1/Hoechst/Mitotraker Cell Viability Assay. YO-PRO-1/Hoechst/Mitotraker triple staining, which was used to count apoptotic cells, can differentiate between apoptotic and necrotic cells (5). Hoechst 33342 freely enters living cells and, therefore, stains the nuclei of viable cells, as well as those that have suffered apoptosis or necrosis. Apoptotic cells can be distinguished from viable and necrotic cells on the basis of nuclear condensation and fragmentation. YO-PRO-1 is a membrane-impermeant dye that is generally excluded from viable cells, whereas early-stage apoptotic and necrotic cells are YO-PRO-1-positive. Cells were plated at a density of 5×10^5 cells/chamber in culture slides (BD Biosciences) and treated for 48 h with the indicated concentrations of PU-H71. Cells were washed with DME-HG and then immersed in DME-HG supplemented with 10% FBS with added YO-PRO-1 (Invitrogen) at 1 μM and Hoechst-33342 (Sigma-Aldrich) at 8 μM. Cell viability was measured 30 min later using a Leica TCS SP2 AOBS laser-scanning confocal microscope. In a blind manner, a total of at least 400 cells per condition were counted, and cell mortality was quantified by expressing the number of YO-PRO-1-positive cells (λ_{ex} 491 nm, $\lambda_{em} > 509$ nm) as a percentage of the number of Hoechst 33342-positive cells. Images were collected at 100× magnification.

Acridine Orange/Ethidium Bromide Cell Viability Assay. The Easycount ViaSure kit (Immunocon) was used in conjunction with the Easycount system to count dead and live cells automatically. The ViaSure Staining Reagent uses a mixture of ready-to-use nucleic acid dyes, acridine orange and ethidium bromide, to identify live and dead cells, respectively, in a single test. Acridine orange is taken up by both viable and nonviable cells and emits green fluorescence if intercalated into double-stranded nucleic acid (DNA) or red fluorescence if bound to single-stranded nucleic acid (RNA). Ethidium bromide is taken up only by nonviable cells and emits red fluorescence by intercalation into DNA (1). Viable cells have uniform green nuclei with organized structure (2). Early apoptotic cells (which still have intact membranes but have started to undergo DNA cleavage) have green nuclei, but perinuclear chromatin condensation is visible as bright green patches or fragments (3). Late apoptotic cells have orange to red nuclei with condensed or fragmented chromatin (4). Necrotic cells have a uniformly orange to red nuclei with organized structure.

Caspase-3,7 Activity. Cells were plated and treated as described in the growth inhibition assay section. Following a 24-h exposure of cells to Hsp90 inhibitors, 100 μL buffer containing 10 mM Hepes, pH 7.5, 2 mM EDTA, 0.1% CHAPS, 0.1 mg/mL PMSF, Complete Protease Inhibitor mix (#1697498; Roche), and the caspase substrate Z-DEVD-R110 (#R22120; Molecular Probes) at 25 μM was added to each well. Plates were placed on an orbital shaker to promote cell lysis and reaction. The fluorescence signal of each well was measured in an Analyst GT (Molecular Devices) microplate reader (excitation 485 nm; emission at 530 nm). The percentage increase in caspase-3,7 activity was calculated by comparison of the fluorescence reading obtained from treated versus control cells.

Caspase-Regulated Cell Death. The activity of caspase-7 and caspase-3 was determined using the Apo-ONE caspase 3/7 assay (Promega) following the manufacturer's instructions. Briefly, MDA-MB-468 cells at the exponential phase of growth were exposed for 2 h to 40 μM of the Caspase Inhibitor 1 (Calbiochem) followed by a treatment for 18 h with PU-H71, 1 μM. The pro-fluorescent Z-DEVD-R110 substrate was added to the cell culture, and cells were incubated for 1 h. The activation of caspases 3 and/or 7 caused the peptide DEVD to be removed from Z-DEVD-R110 allowing the R110 group to become intensely fluorescent ($Ex_{499\ nm}/Em_{521\ nm}$). The fluorescence was determined using the Synergy4 microplate reader (BioTek Instruments). Viability was determined using a fluorometric resazurin reduction method (CellTiter-Blue; Promega) following the manufacturer's instructions. The fluorescence (Ex_{560nm}/Em_{590nm}) was determined using the Synergy4 microplate reader. The number of viable cells in each treated well was calculated by using the linear least-squares regression of the standard curve.

The cell viability was verified by the Trypan-blue exclusion method.

NF-κB Transcriptional Activity Assay. NF-κB activity in MDA-MB-231 cells treated for 24 h with 0.5 and 1.0 μM PUH71 was measured using a colorimetric TransAM NF-κB p65 Transcription Factor Assay kit (Active Motif). Following treatment exposure, cells were washed once, then scraped, and collected in 3 mL ice-cold PBS/phosphatase-inhibitor buffer (PIB) supplied in the kit. Cells were pelleted and then lysed on ice for 15 min with 1 mL ice-cold hypotonic buffer provided in the kit. Nonidet P-40 was added (0.5%, vol/vol), and the crude lysate was microcentrifuged; the nuclear pellet was resuspended in complete lysis buffer and incubated for 30 min on a rocking platform at 4ŸC. The nuclear suspension was then microcentrifuged for 10 min at 14,000 × *g* at 4 °C, and the supernatant was used as the nuclear extract. Two micrograms protein per sample in triplicate was used to measure NF-κB activity in a 96-well plate, precoated with an oligonucleotide containing the NF-κB p65 subunit consensus binding site (6). The active NF-κB present in MDA-MB-231 nuclear extracts binds to this consensus oligonucleotide, and NF-κB activity was measured as optical density units read at 450 nm.

Cell Invasion Assay. The invasion capacity of MDA-MB-231 cells was examined using a Boyden chamber Matrigel invasion assay. MDA-MB-231 cells platted at 5×10^5 cells/well in a 6-well plate (Becton Dickinson), were pretreated for 24 h with DMSO, PU-H71 (0.5 and 1 μM), Akti (50 μM), ERK2i (5 μM), Tyk2i (100 μM), and PKCα/βi (25 and 100 nM). Cell viability was estimated by trypan blue exclusion. In summary, cells were stained with tryptan blue and counted manually with a hemacytometer. Viable cells, which are cells that exclude tryptan blue, were washed 3 times with serum-free DMEM and resuspended in 0.3 mL serum-free DMEM. Equal numbers of viable cells (2×10^5) were added to the upper compartment of the Boyden chamber, and treatment medium containing 10% FBS in DMEM was added to the lower chamber. Boyden chambers contained an 8-μm PET track-etched membrane, coated with Matrigel transwells (BD Biosciences). After cells were incubated for 20 h at 37 °C, cells invading to the lower side of the membrane were fixed with were fixed in 100% methanol for 2 min, stained in 0.5% crystal violet for 2 min, rinsed in water, and examined under a bright-field microscope. Cells in 10 fields per membrane were counted at 100× objective.

Proteasome-Mediated Degradation. MDA-MB-468 cells were pretreated with 10 μM MG132 (Sigma-Aldrich) for 2 h before the addition of 1 μM PU-H71. Cells were collected at 24 h and lysed in 50 mM Tris, pH 7.4, 150 mM NaCl, and 1% Nonidet P-40 lysis buffer. Nonidet P-40 insoluble fractions were lysed in 50 mM Tris, pH 7.4, and 2% SDS, and boiled for 15 min. Proteins were separated by SDS/PAGE followed by a standard Western blotting procedure. Blots were visualized by autoradiography using enhanced chemiluminescence detection system (GE Healthcare).

Chemical Precipitation and Proteomics. Agarose beads with immobilized PU-H71 or an Hsp90-inactive chemical (ethanolamine) were washed 3 times with PBS. Beads were incubated overnight at +4 °C with cellular lysates dissolved in 20 mM Tris-HCl, pH 7.4, 25 mM NaCl, 20 mM Na_2MoO_4, 0.1% Nonidet P-40, 10 μg/mL aprotinin, and 10 μg/mL leupeptin, then washed 5 times with the above buffer. For Western blot analyses, proteins were eluted with SDS-containing buffer, separated by gel electrophoresis and analyzed by immunobloting. For proteomic analyses, samples were subjected to in-gel digestion as described before (7), and subjected to liquid chromatography-tandem mass spectrometry (LC-MS/MS) analyses at the Proteomics Facility of Weill Cornell Medical College (WCMC) or at the Rockefeller University Proteomics Core. In brief, after washing with water, the gel slices are cut into 1-mm^3 pieces, reduced with 10 mM DTT in 100 mM NH_4HCO_3 at 56 °C for 30 min, alkylated with a 55 mM iodacetamide solution in 100 mM NH_4HCO_3 at room temperature in the dark for 20 min, and then digested with trypsin (13 ng/μL) at 37 °C overnight. Peptides are extracted with 100–200 μL 66.6% acetonitrile/5% formic acid, and the volume of combined peptide extract is reduced to approximately 10 μL in a SpeedVac before MS analysis. LC-MS/MS analyses at WCMC is performed using an 1100 series LC coupled to an XCT plus ion trap mass spectrometer (Agilent Technologies). The system is equipped with an Agilent Chip Cube interface and a silicon wafer "chip-column" that integrates a C18 enrichment column, C18 resolving column, and nanospray emitter. In-gel protein digests are loaded and desalted on the enrichment column at a flow rate of 4 μL/min and then resolved at a flow rate of 0.35 μL/min on a 40 mm × 75 μM ZORBAX 300 C18 column (Agilent Technologies). The LC gradient is 3% to 45% Solvent B for 25 min, followed by 45% to 90% Solvent B for 5 min. Mobile phase solvent A is 0.1% formic acid in 3% ACN, and Solvent B is 0.1% formic acid in 90% ACN. Mass spectra are acquired in positive-ion mode with automated data-dependent MS/MS on the 4 most intense ions from precursor MS scans. *SPECTRUM MILL* software (Agilent Technologies) or Mascot was used to process LC-MS/MS raw data and to search protein database for protein identification.

Animal Studies. Four- to 6-week-old *nu/nu* athymic female mice were obtained from Taconic Farms. Experiments were carried out under an Institutional Animal Care and Use Committee-approved protocol, and institutional guidelines for the proper and humane use of animals in research were followed. MDA-MB-468 (1×10^7 cells), HCC-1806 (1×10^6 cells), or MDA-MB-231 (2×10^7 cells) were s.c. implanted in the right flank of mice using a 20-gauge needle and allowed to grow. Before administration, a solution of PU-H71 was formulated in PBS (pH 7.4). All mice received Augmentin (amoxicillin/clavulanate potassium; SmithKline Beecham) in their drinking water while on therapy. Mice were killed by CO_2 euthanasia.

Pharmacodynamic (PD) and Pharmacokinetic (PK) Studies. For PD and PK assays, tumors were allowed to reach 6–8 mm in diameter before treatment. Mice (n = 2) bearing MDA-MB-468, HCC-1806, or MDA-MB-231 tumors were administered i.p. 75 mg/kg PU-H71. Animals were killed by CO_2 euthanasia at 6, 12, 18, 24, 36, and 48 h postadministration of PU-H71. At sacrifice, tumor, kidney, liver, heart, lung, and brain tissue were collected. A gross necropsy was performed on all mice. For quantitative drug analysis, weighted pieces of tumor and normal tissue samples were homogenized in acetonitrile/methanol (3:1) solution at a 1:3 wt/vol ratio. PU-H71 was extracted in methylene chloride, and the organic layer was separated, speedily dried under vacuum, and reconstituted in the mobile phase. Concentrations of PU-H71 were determined by high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) at the Analytical Core Facility of the Memorial Sloan-Kettering Cancer Center. Haloperidol was added as the internal standard. Compound analysis was performed in the API 4,000 LC/MS/MS system (Applied Biosystems), which was coupled with a Shimadzu LC system and a 96-well plate autosampler. A Gemini C_{18} column was used for the LC separation. The analyte was eluted under an isocratic condition for 4 min at a flow rate of 0.4 mL/min. For protein analysis, tumors were homogenized in SDS lysis buffer (50 mM Tris, pH 7.4, 2% SDS) and subjected to Western blot analysis. For immunohistochemical analysis, tumors were harvested and further paraffin-embedded.

Imaging Mass Spectrometry. MDA-MB-468 xenografted tumors were established as described above. A single dose of 50 mg/kg PU-H71 was administered, and tumor, liver, and brain were removed following sacrifice at 0.5, 3, 4, 6, and 8 h postdose. Excised tumors and organs were flash-frozen in a cryo vial by submerging in liquid nitrogen. Tissues were sliced to a thickness of approximately 12 μm using a Leica Microsystems CM 3050 cryomicrotome held at -25 °C and thaw-mounted to stainless steel MALDI target plates. A (20 mg/mL) α-cyano-hydroxycinnamic acid (in 60% acetonitrile with 0.1% trifluoroacetic acid) was used for matrix. Plate-mounted tissues were spray-coated with matrix solution using a TLC sprayer. Typically 20 to 30 coating cycles were applied. Images were acquired using a quadrupole-TOF tandem mass spectrometer with an orthogonal MALDI source attached (QSTAR Elite; Applied Biosystems/MDS Sciex). MALDI Images were acquired at a pixel resolution of 150 μm $\times$ 150 μm from a Nd:YAG laser operated at 2.5 μJ, 1,000 Hz with a 0.5 s accumulation time per image spot. Collisional activation of selected ions was carried out using relative collision energy of 25 V with argon as collision gas. Mass spectrometric data were processed using MALDI Server 5.0 software provided by Applied Biosystems/MDS Sciex, and images were visualized using BioMAP 3.7.2 software (Novartis).

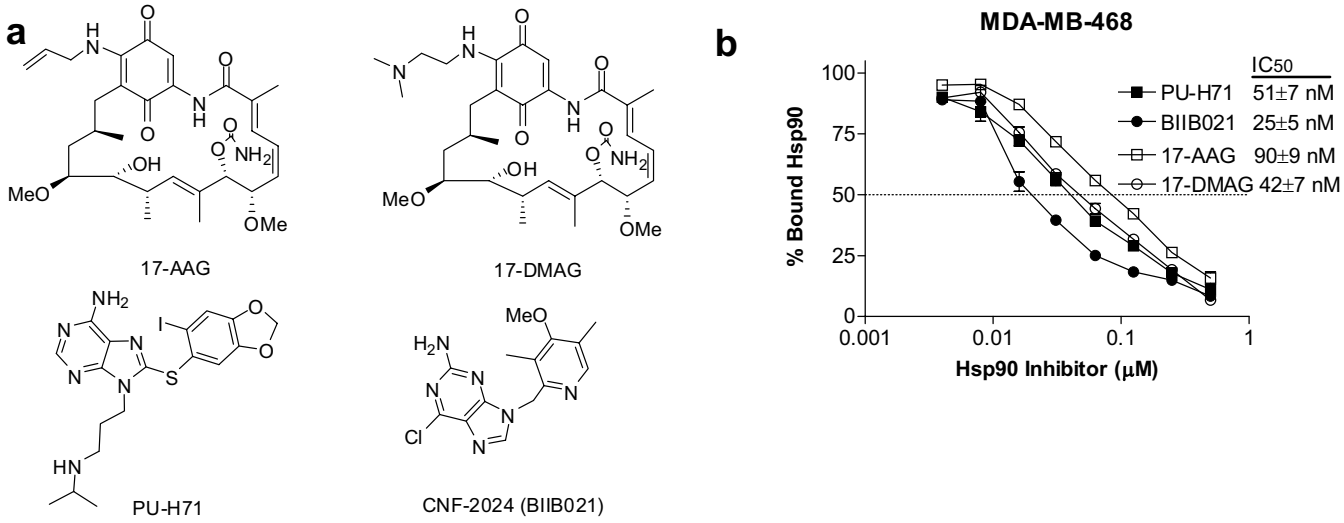
Apoptotic Index. The DNA fragmentation (single-stranded and double-stranded) coupled to the apoptotic response was detected in morphologically identifiable nuclei and apoptotic bodies present in formalin-fixed paraffin-embedded tumors by the TUNEL assay (ApopTag; Chemicon) (8). Briefly, deparaffinized slides were pretreated for 15 min with 0.5% trypsin or pepsin when strong digestion was required (Zymed), to improve the exposure of DNA. Endogenous peroxidase was quenched using 3% hydrogen peroxide (Sigma-Aldrich) in PBS for 5 min followed by pH equilibration and incubation with TdT enzyme for 1 h. Then, anti-digoxigenin-peroxidase was applied to the slides. Color was developed with diaminobenzoate chromogen peroxidase substrate (Vector) and counterstained with methyl green (Fisher) 0.5% in sodium acetate pH 4.0. Specimens were treated with 1-butanol, dehydrated in xylene (Fisher), and mounted (Vectamount; Vector).

Immunohistochemistry (IHC). Phospho-AKT and phospho-histone3 were identified by IHC. Briefly, deparaffinized slides were antigen-retrieved in citrate buffer, pH 6.0 (Zymed), then 1:1,000 rabbit anti-phospho-AKT (R&D Systems) and rabbit phospho-histone3 (Upstate Biotechnologies) were applied, followed by incubation with a corresponding biotinylated-conjugated secondary antibody (Vector). Slides were incubated with a preformed avidin and biotinylated horseradish peroxidase macromolecular complex, Vectastain ABC (Vector). Color was developed with diaminobenzoate chromogen peroxidase substrate (Vector). IHC results were scored using ImageJ software (National Institutes of Health).

Efficacy Studies. Mice bearing MDA-MB-468 tumors reaching a volume of 100–150 mm^3 were treated i.p. (i.p.) using different doses and schedules: Group 01 (n = 8) PBS; group 02 (n = 8) PU-H71 at 50 mg/kg on alternate days; group 03 (n = 8) PU-H71 at 50 mg/kg 5xqd; group 04 (n = 8) PU-H71 at 75 mg/kg 3$\times$ week; group 05 (n = 8) PU-H71 at 75 mg/kg on alternate days. Mice bearing HCC-1806 or MDA-MB-231 xenografted tumors received PU-H71 at 75 mg/kg on alternate days. Tumor volume was determined by measurement with Vernier calipers, and tumor volume was calculated as the product of its length $\times$ width^2 $\times$ 0.4. Tumor volume was expressed on indicated days as the median tumor volume $\pm$ SD indicated for groups of mice. Percent (%) tumor growth inhibition values were measured on the final day of study for drug-treated compared with vehicle-treated mice and are calculated as $100 \times \{1 - [(\text{Treated}_{\text{Final day}} - \text{Treated}_{\text{Day 1}})/(\text{Control}_{\text{Final day}} - \text{Control}_{\text{Day 1}})]\}$. Tumor regression values were determined by calculating the ratio of median tumor volumes at the time when treatment was initiated to median tumor volume on the final day of study for a given treatment group. Percent (%) tumor regression is $100 \times [1 - (\text{Treated}_{\text{Final day}}/\text{Treated}_{\text{Day 1}})]$.

Statistical Analysis. Data were analyzed by unpaired 2-tailed *t* tests as implemented in GraphPad Prism (version 4; GraphPad Software).

1. Chiosis G, Caldas Lopes E, Solit D (2006) Heat shock protein-90 inhibitors: A chronicle from geldanamycin to today's agents. *Curr Opin Investig Drugs* 7:534–541.
2. Rodina A, et al. (2007) Selective compounds define Hsp90 as a major inhibitor of apoptosis in small cell lung cancer. *Nat Chem Biol* 3:498–507.
3. Du Y, et al. (2007) High-throughput screening fluorescence polarization assay for tumor-specific Hsp90. *J Biomol Screen* 12:915–924.
4. He H, et al. (2006) Identification of potent water-soluble purine-scaffold inhibitors of the heat shock protein 90. *J Med Chem* 49:381–390.
5. Idziorek T, Estaquier J, De Bels F, Ameisen JC (1995) YOPRO-1 permits cytofluorometric analysis of programmed cell death (apoptosis) without interfering with cell viability. *J Immunol Methods* 185:249–258.
6. Renard P, et al. (2001) Development of a sensitive multi-well colorimetric assay for active NFkappaB. *Nucleic Acids Res* 29:E21.
7. Shevchenko A, Tomas H, Havlis J, Olsen JV, Mann M (2006) In-gel digestion for mass spectrometric characterization of proteins and proteomes. *Nat Protocols* 1:2856–2860.
8. Gavrieli Y, Sherman Y, Ben-Sasson S (1992) Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J Cell Biol* 119:493–501.



c

	PU-H71	CNF-2024 (BIIB021)	17-AAG	17-DMAG
MDA-MB-468	65±8 nM	69±5 nM	512±15 nM	31±5 nM
MDA-MB-231	140±5 nM	210±13 nM	810±18 nM	33±6 nM
HCC-1806	87±3 nM	152±11 nM	163±14 nM	42±7 nM

Fig. S1. Hsp90 inhibitors and their biological activity in TNBC cells. (*a*) Chemical structure of representative Hsp90 inhibitors. (*b*) Their apparent affinity for Hsp90 from MDA-MB-468 cell extracts was determined as described in *SI Materials and Methods*. Drugs were assayed in triplicate. Error bars represent the SD of the mean. (*c*) Representative TNBC cells were incubated with increasing concentrations of PU-H71, BIIB021, 17-AAG, and 17-DMAG Hsp90 inhibitors, their effects of cell growth assessed over 72 h, half inhibitory concentrations (IC_{50}s) recorded, and data tabulated.

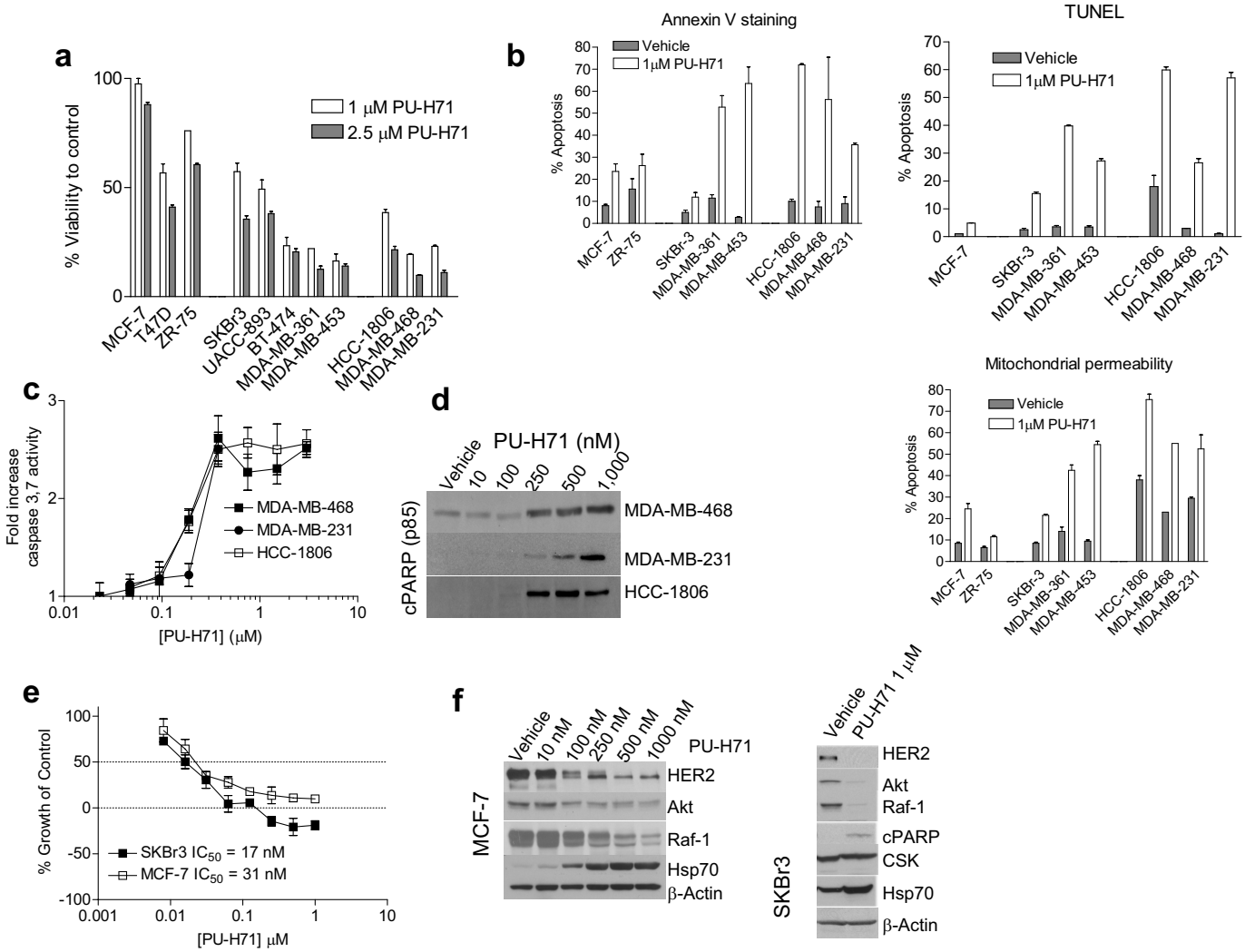


Fig. S2. The effect of PU-H71 on (*a*) cell viability and (*b–d*) apoptosis in breast cancer cells. (*a*) The ER$^+$ cells MCF-7, T47D, and ZR-75, the HER2$^+$ cells SKBr3, UACC-893, BT-474, MDA-MB-361, and MDA-MB-453, and the TNBC cells HCC-1806, MDA-MB-468, and MDA-MB-231 were treated for 48 h with the indicated concentrations of PU-H71 and cell viability determined by acridine orange/ethidium bromide staining. (*b*) A select panel of ER$^+$, HER2$^+$, and TNBC cells were treated for 48 h with PU-H71 (1 μM) and percent cells in early and late apoptosis detected by FITC-annexin V and dUTP nick end-labeling (TUNEL), respectively, whereas cells with depolarized mitochondrial membrane were detected as described in *SI Materials and Methods*. (*c*) TNBC cells were treated for 24 h with increasing concentrations of PU-H71. Caspase-3,7 activity was a measure of PU-H71 potency in cleaving the caspase substrate Z-DEVD-R110 and releasing rhodamine. Caspase-3 activity in vehicle-treated cells is normalized to 100%. Drugs were assayed in triplicate. Error bars represent the SD of the mean. (*d*) TNBC cells were treated for 24 h with increasing concentrations of PU-H71. PARP cleavage was determined by Western blot analysis. (*e*) The ER$^+$ MCF-7 and the HER2$^+$ SKBr3 cells were incubated with increasing concentrations of PU-H71 and its effect of cell growth assessed over 72 h. The half inhibitory concentration (IC_{50}) was recorded. (*f*) The effect of PU-H71 on oncoproteins in the ER$^+$ MCF-7 and the HER2$^+$ SKBr3 cells. Cells were treated for 24 h with the indicated concentrations of PU-H71, and protein extracts were subjected to immunoblotting. β-actin was used to assess the quality of total protein loading.

a

Exp#1	Exp#2	Exp#3	Exp#4	Database Accession #	%AA Coverage	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	Proliferation/invasion
1	3	3	3	P28482	27	5	53.72	Mitogen-activated protein kinase 1
9.53E-07	1.32E+08	1.93E+08	1.48E+08					ERK2, PRKM1, PRKM2
1	1	3	1	Q15418	5	2	19.54	p90S6K MAP kinase-activated protein kinase 1a
6.25E-07	8.56E+07	1.82E+08	1.09E+08					
1	0	0	1	P05771	10	2	11.67	Protein kinase C beta type
5.45E+08	0.00E+00	0.00E+00	1.20E+08					
1	0	0	1	Q9ULU4	4	2	15.18	Protein kinase C-binding protein 1
3.33E+08	0.00E+00	0.00E+00	1.36E+07					
1	5	8	8	P63244	47	9	80.53	Receptor of activated protein kinase C 1
9.62E-07	2.44E+08	2.82E+08	2.81E+08					RACK1
0	1	0	1	P29597	1	1	7.5	Non-receptor tyrosine-protein kinase TYK2
0.00E+00	4.24E+08	0.00E+00	5.81E+08					

Serine/threonine-protein kinase TBK1	TAK1-binding protein 3 (Tab3)
K.MLHLR.K	K.QLKLEK.E
K.EEMEGVVK.E	R.RISVTSK.V
K.MFTASSGIK.H	K.QGSHSVAYLPYTASSLSK.G
R.VIGEDGQSVYK.L	K.YLYMEYHSPDDNRMNR.N
R.YDLDGDASMAK.A	R.SAAPEPIQPISVIPGSGGEKGSHK.Y
R.LAYNEEQIHK.F	R.GNFDPKAMNNFYDNIEPGPVVPPKPSK.K
K.DDYNETVHKK.T	
K.IITGKPSGAISGVQK.A	**TAK1-binding protein 2 (Tab2)**
R.MVIHVFSLQQMTAHK.I	R.ELEIQKK.K
R.LAQHFPKTTEENPIFVVSR.E	R.VVVTQPNTK.Y
R.DIKPGNIMRVIGEDGQSVYK.L	R.LQRELEIQK.K
- .MQSTSNHLWLLSDILGQGATANVFRGR.H	K.SRAIGNNSATSPR.V
K.MFTASSGIKHTMTPIYPSSNTLVEMTLGMK.K	R.QKFPEVPEVVVSR.C 25863 25899 25901
K.MFTASSGIKHTMTPIYPSSNTLVEMTLGMK.K	R.NQPTLFISTNSGASAASR.N
K.IITGKPSGAISGVQKAENGPIDWSGDMPVSCSLSR.G	R.SNSISQIPSLEEMQQLR.S
	R.SNSISQIPSLEEMQQLRSCNR.Q
Interleukin-1 receptor-associated kinase 1 (IRAK-1)	
K.LGDFGLAR.F	
K.ENADLEWTAVK.Q	
K.QSFLTEVEQLSR.F	
K.IGEGGFGCVYRAVMR.N	
R.GPNQPVESDESLGGLSAALR.S	
K.VMDALEPADWCQFAALIVRDQTELR.L	
R.GPNQPVESDESLGGLSAALRSWHLTPSCPLDPAPLR.E	

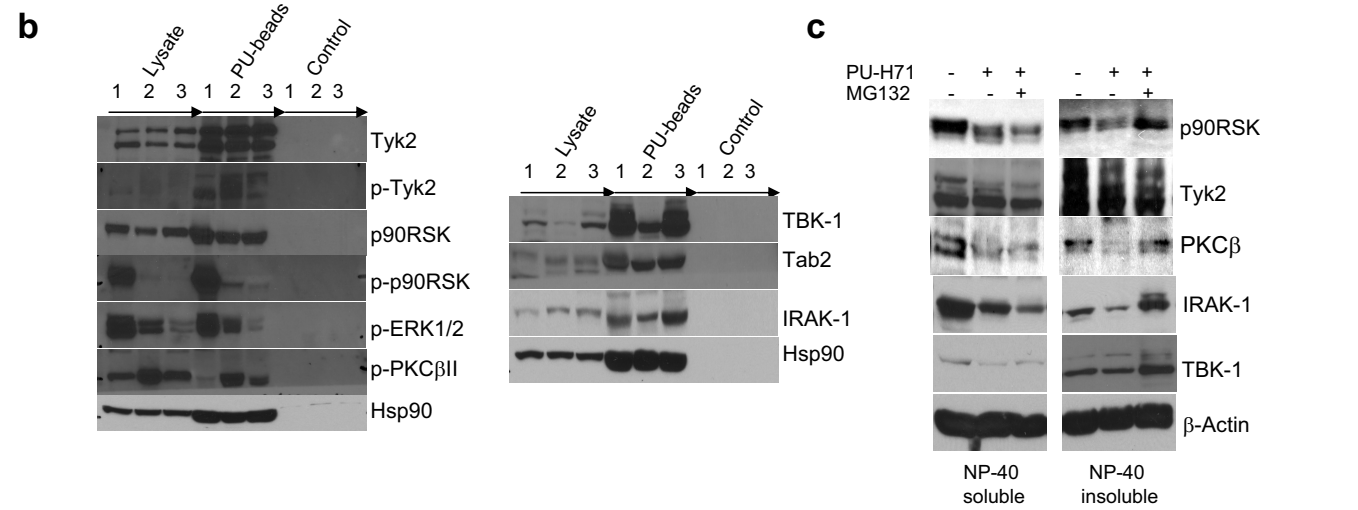


Fig. S3. Hsp90 onco-clients identified in TNBC cells by PU-H71-beads pull-downs. (*a*) Proteomics analyses were conducted as described in *SI Materials and Methods*. *SPECTRUM MILL* software (Agilent) and *Mascot* were used to process LC-MS/MS raw data of 4 experiments and to search protein database for protein identification. (*b*) Proteomics findings were validated by Western blot. Hsp90-containing protein complexes isolated through chemical precipitation with beads having attached PU-H71 (PU-beads) or an Hsp90-inert molecule (control), and the endogenous protein content of these TNBC cells (lysate) was analyzed by Western blot. 1, MDA-MB-468; 2, MDA-MB-231; and 3, HCC-1806 cells. (*c*) Degradation of the Hsp90 clients is partly mediated by the proteasome. Cells were treated for 24 h with vehicle, PU-H71 (1 μM) or pretreated for 2 h with the proteasome inhibitor MG132 (10 μM) before the addition of PU-H71. Proteins in the soluble and insoluble fractions were analyzed by Western blot as described in *SI Materials and Methods*.

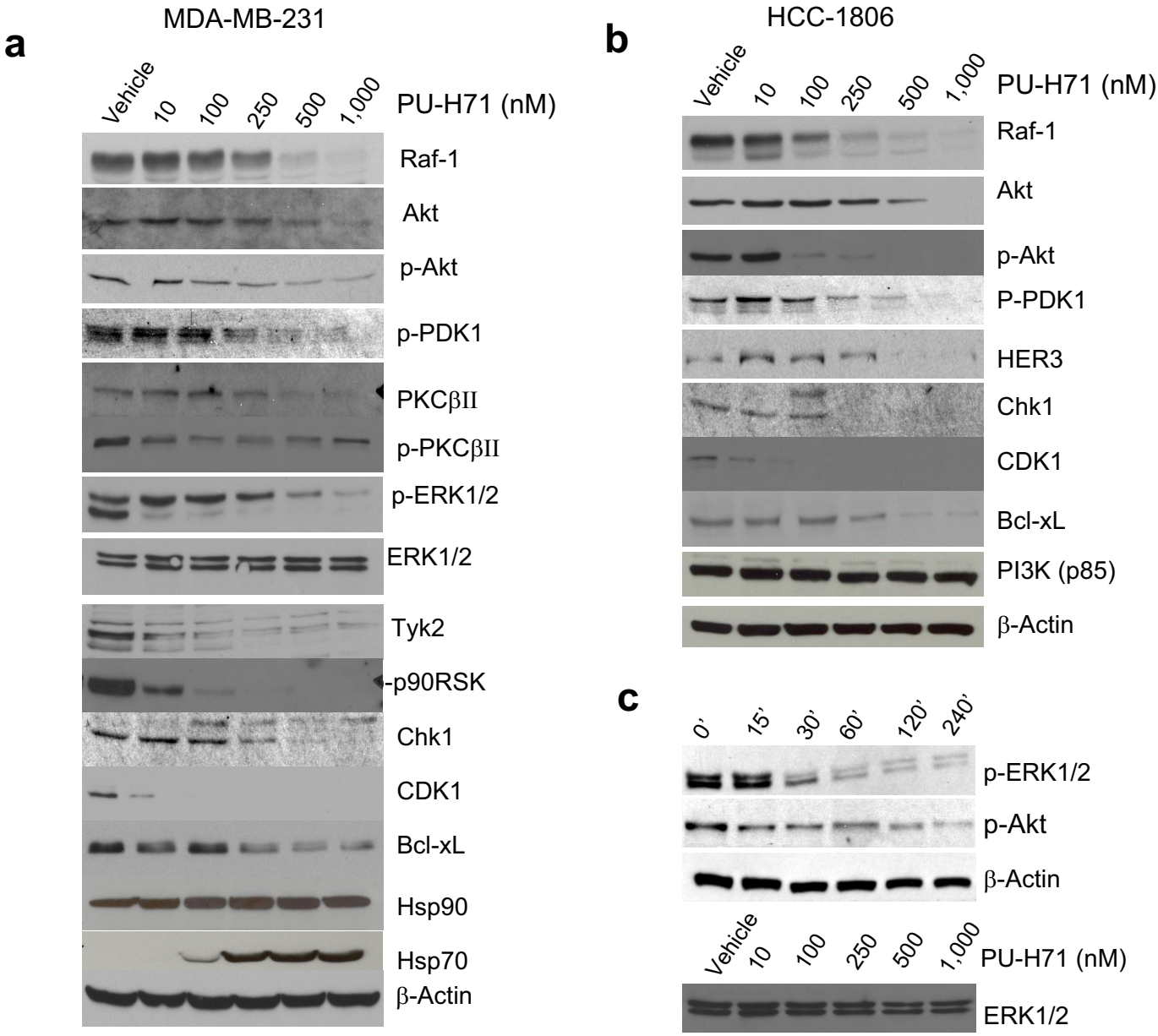


Fig. S4. Oncoproteins down-regulated by PU-H71 in TNBC cells. MDA-MB-231 cells (*a*) and HCC-1806 cells (*b*) were treated for 24 h with increasing concentrations of PU-H71, and protein extracts were subjected to immunoblotting. PI3K (p85) and β-actin were used to assess the quality of total protein loading. (*c*) MDA-MB-468 cells were treated with PU-H71 (1 μM) for the indicated times or for 24 h with the indicated concentrations. Proteins were analyzed by Western blot.

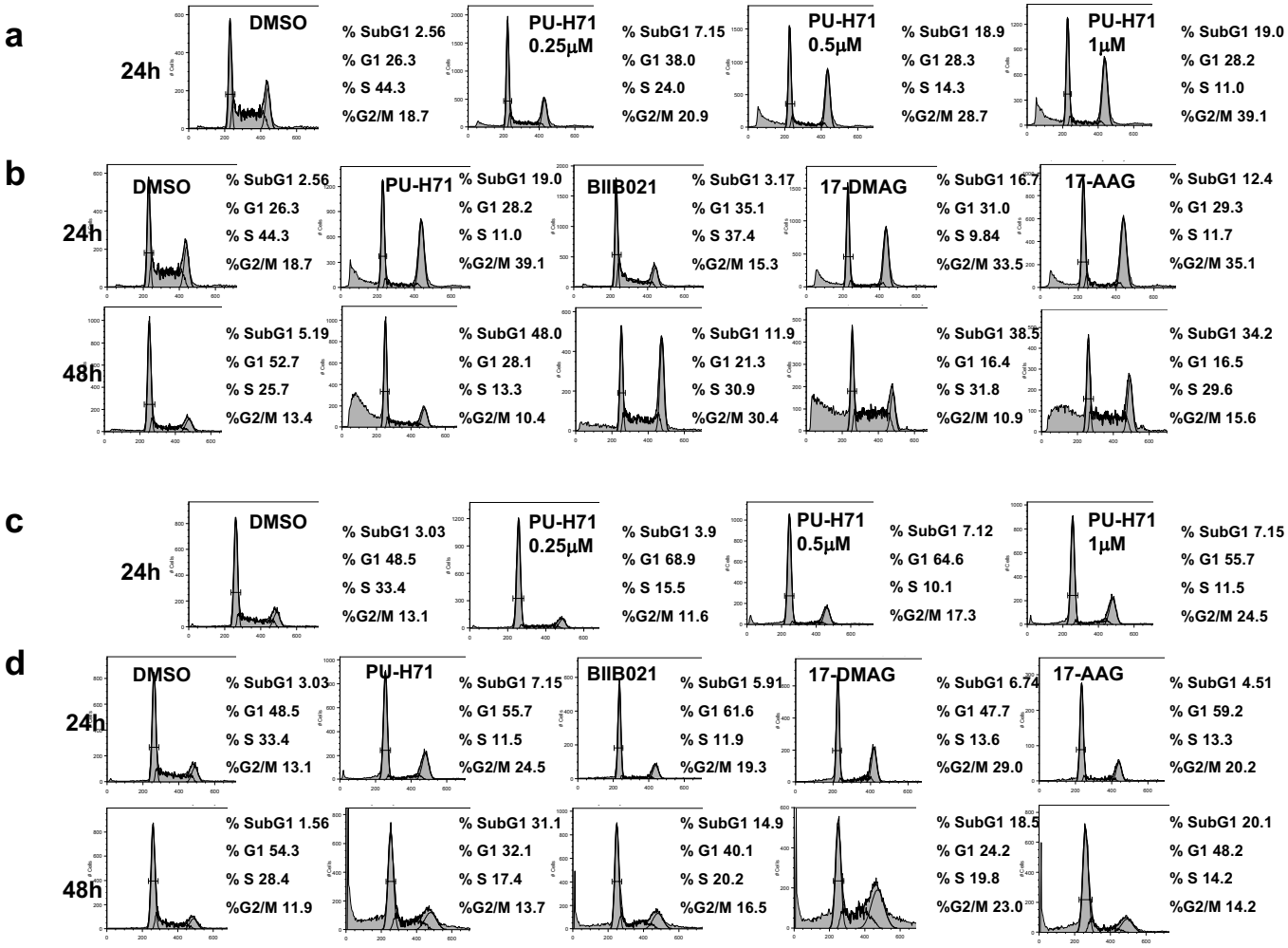


Fig. S5. The effect of Hsp90 inhibitors on cell cycle in HCC-1806 and MDA-MB-231 cells. (*a*) HCC-1806 cells were treated for 24 h with vehicle or with the indicated concentrations of PU-H71 or (*b*) for 24 h (*Upper*) or 48 h (*Lower*) with vehicle or with the indicated Hsp90 inhibitors (1 μM). DNA content was analyzed by propidium iodide staining. (*c*) MDA-MB-231 cells were treated for 24 h with vehicle or with the indicated concentrations of PU-H71 or (*d*) for 24 h (*Upper*) or 48 h (*Lower*) with vehicle or with the indicated Hsp90 inhibitors (1 μM). DNA content was analyzed by propidium iodide staining.

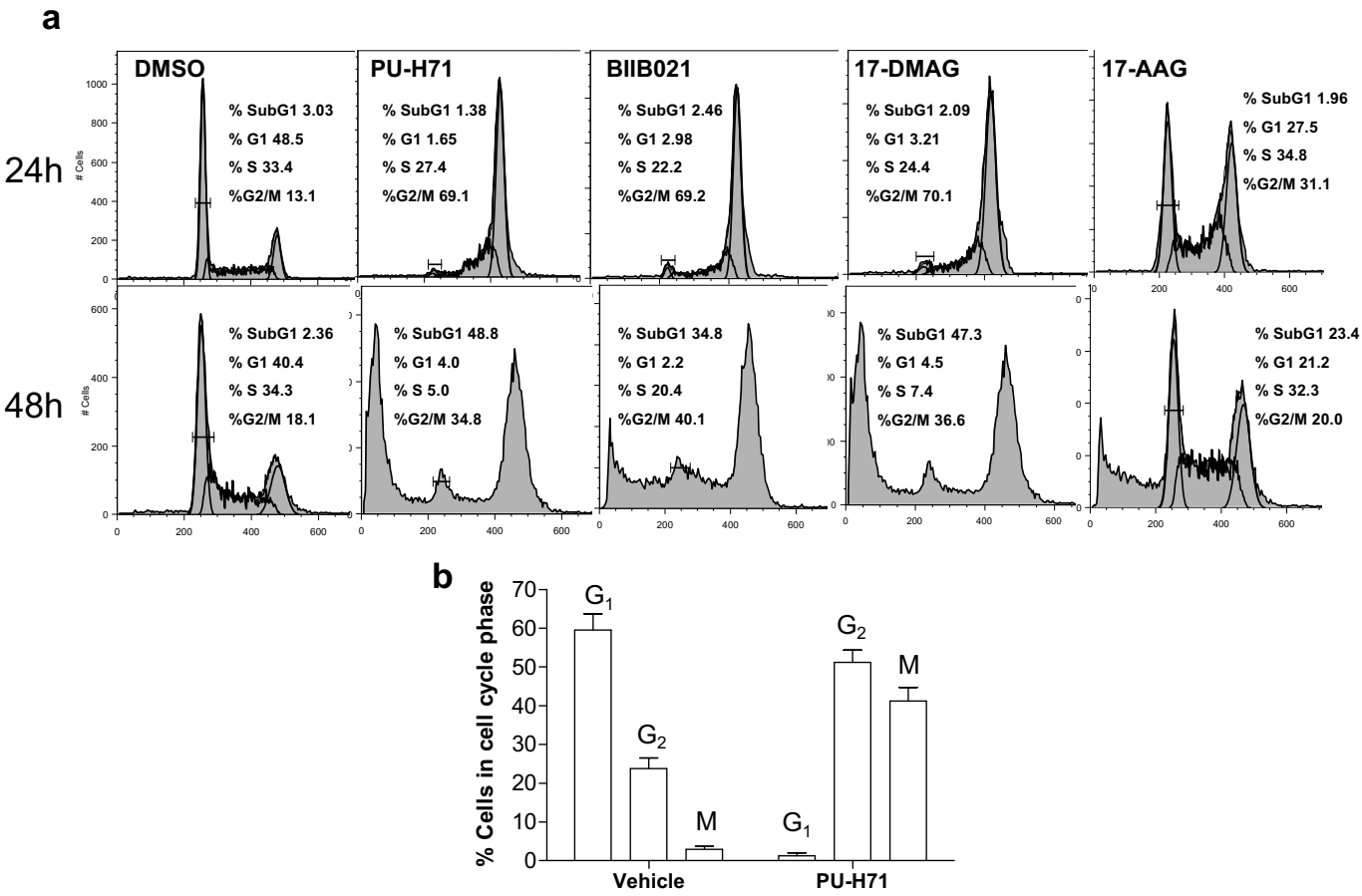


Fig. S6. The effect of Hsp90 inhibitors on cell cycle in MDA-MB-468 cells. (*a*) MDA-MB-468 cells were treated for 24 h (*Upper*) and 48 h (*Lower*) with vehicle or with the indicated Hsp90 inhibitors (1 μM). DNA content was analyzed by propidium iodide staining. (*b*) PU-H71 blocks TNBC cells in G2-M. MDA-MB-468 cells were treated for 24 h with vehicle or PU-H71 (1 μM). Mitotic cells were identified using an antibody against phosphohistone H3. DNA content was analyzed by propidium iodide staining.

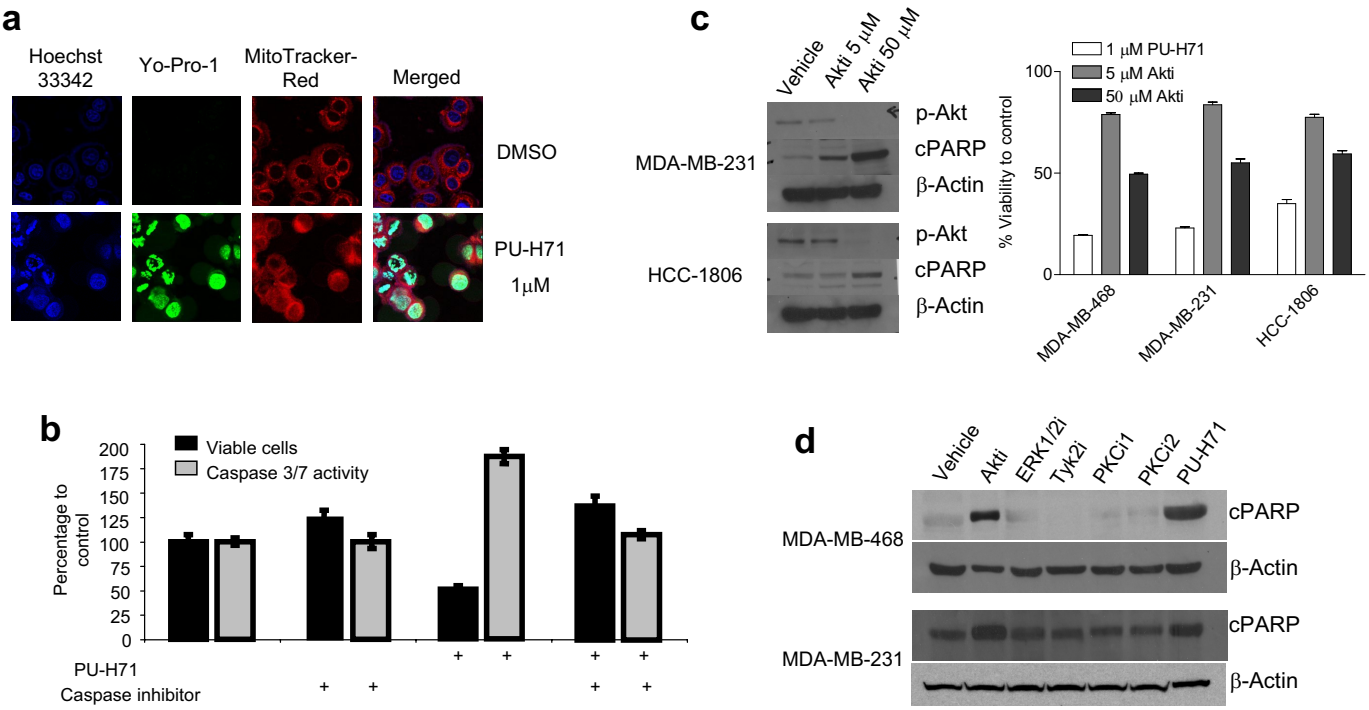


Fig. S7. Induction of cell death by PU-H71 occurs mainly through apoptosis. (*a*) Representative fluorescence microscopy showing nuclear staining for Hoechst 33342 (blue), YO-PRO-1 (green), and MitoTracker Red (red) of MDA-MB-468 cells treated for 48 h with vehicle (*Upper*) or PU-H71 (1 μM) (*Lower*). (*b*) Addition of PU-H71 (1 μM) to MDA-MB-468 cells for 18 h increased the activity of caspases 3/7 and consequently decreased the number of viable cells. This effect was overcome by the addition of a pan-caspase inhibitor, Z-VAD(OMe)-FMK, before PU-H71 treatment. The activity of caspase-3,7 was determined using the Apo-ONE caspase 3/7 assay (Promega) following the manufacturer's instructions. Briefly, MDA-MB468 cells at the exponential phase of growth were exposed for 2 h to 40 μM Caspase Inhibitor 1 (Calbiochem), followed by a treatment for 18 h with PU-H71 1 μM. Viability was determined using a fluorometric resazurin reduction method (CellTiter-Blue; Promega). (*c*) TNBC cells were treated for 24 h with the indicated concentrations of Akt inhibitor and proteins analyzed by Western blot (*Left*) or for 48 h and cell viability analyzed by dual acridine orange/ethidium bromide staining (*Right*). (*d*) MDA-MB-468 and MDA-MB-231 cells were treated for 24 h with the indicated inhibitors as described in *Materials and Methods* and proteins analyzed by Western blot.

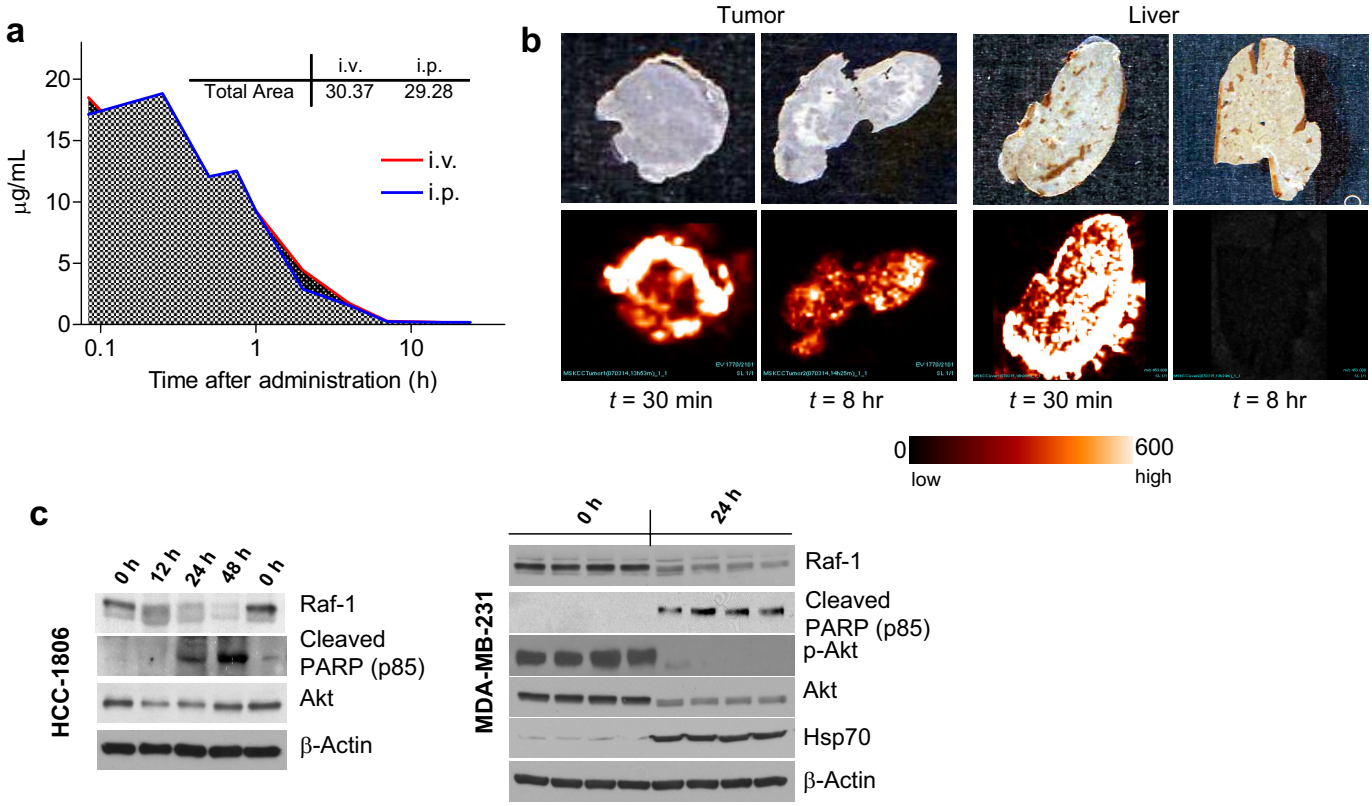
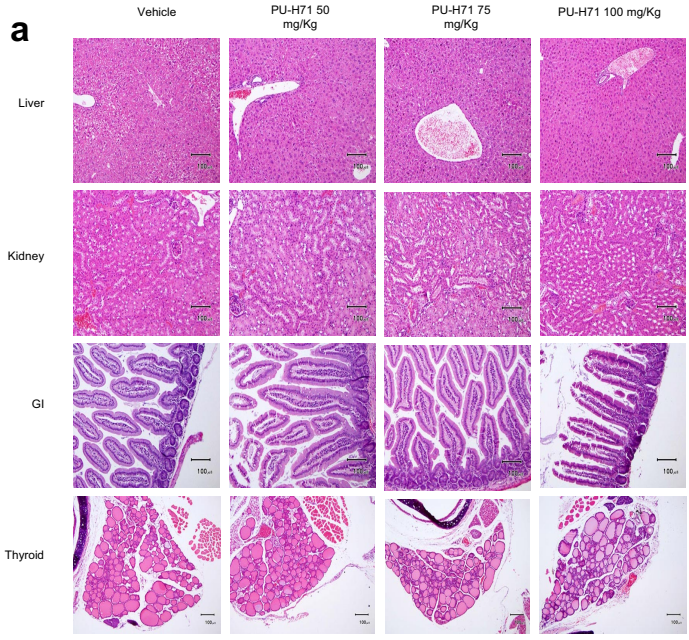
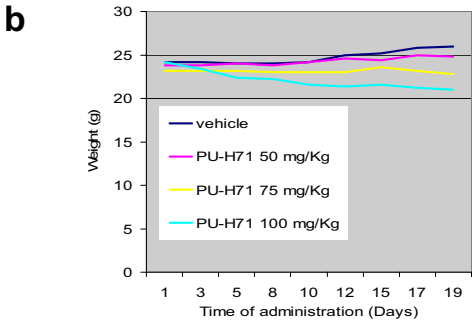


Fig. S8. Pharmacokinetic and pharmacodynamic evaluation of PU-H71 in TNBC. (*a*) B6D2F1 mice were administered i.v. and i.p. 40 mg/kg PU-H71, and drug levels in plasma were detected by LC-MS/MS. Each data point represents the average value recorded from 3 individual mice. Error bars represent the SD of the mean. (*b* and *c*) Xenograft tumors were established by injecting cells with reconstituted basement membrane (Matrigel) in nude athymic (nu/nu) mice. Mice were administered i.p. (ip) one dose of PU-H71 (75 mg/kg) dissolved in PBS, pH 7.4. Animals were killed at the indicated times postadministration, and tumors and organs were harvested. (*b*) Tumors and liver slices were visualized by light microscopy (*Upper*), whereas the spatial distribution and relative abundance of PU-H71 in these samples at the indicated times postadministration was visualized by imaging mass spectrometry (*Lower*). (*c*) Protein extracts from the indicated tumors were subjected to Western blot analysis. β-actin levels were used as a loading control. Data are representative of 2 individual experiments.





c

Parameter	Unit	Ref range	Vehicle	St.Dev	PU-H71 50mg/kg	St.Dev	PU-H71 75mg/kg	St.Dev	PU-H71 100mg/kg	St.Dev
WBC	K/uL	1.8-10.7	9.9	1.6	8.4	1.3	10.8	4.1	7.7	2.6
NEUTROPHILS	K/uL	0.1-2.4	2.2	0.3	2.8	0.8	4.1	2.3	4.0	2.1
LYMPHOCYTES	K/uL	0.9-9.3	6.9	1.5	5.1	1.0	6.0	1.7	3.5	1.5
MONOCYTES	K/uL	0.0-0.4	0.8	0.1	0.4	0.1	0.4	0.3	0.2	0.2
EOSINOPHILS	K/uL	0.0-0.2	0.0	0.0	0.0	0.0	0.2	0.2	0.0	0.0
BASOPHILS	K/uL	0.0-0.2	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0
NE%	%	6.6-38.9	22.1	2.9	33.4	7.3	37.0	8.7	49.2	13.7
LY%	%	55.8-91.6	69.7	3.6	61.3	6.9	57.8	8.7	43.4	14.0
MO%	%	0.0-7.5	8.1	1.7	5.0	1.0	3.8	1.6	2.7	1.8
EO%	%	0.0-3.9	0.1	0.1	0.2	0.1	1.2	1.1	0.5	0.2
BA%	%	0.0-2.0	0.0	0.0	0.1	0.0	0.4	0.4	0.1	0.0
RBC	M/uL	6.36-9.42	10.2	0.9	10.6	0.4	10.0	0.5	9.2	0.2
HB	g/dL	11.0-15.1	13.9	0.6	14.2	0.5	13.8	0.6	12.8	0.5
HCT	%	35.1-45.4	47.6	2.4	48.1	2.1	45.5	1.9	41.0	0.8
MCV	fL	45.4-60.3	46.7	2.0	45.6	1.0	45.7	0.9	44.6	0.7
MCH	Pg	14.1-19.3	13.6	0.9	13.5	0.2	13.7	0.3	13.9	0.3
MCHC	g/dL	30.2-34.2	29.1	0.9	29.6	0.4	30.3	0.4	31.3	0.9
RDW	%	12.4-27.0	17.7	0.9	17.2	0.3	16.6	3.3	17.9	1.2
PLT	K/uL	592-2972	742.3	411.1	993.2	143.8	1106.6	123.2	3349.8	436.7
MPV	fL	5.0-20.0	4.4	0.1	4.5	0.1	4.9	0.3	5.3	0.2
ALP	IU/L	23-181	131.2	30.4	156.0	63.1	128.2	49.6	134.2	31.5
ALT (SGPT)	IU/L	16-58	105.8	65.3	51.8	22.9	50.2	28.4	63.2	31.4
AST (SGOT)	IU/L	36-102	195.4	68.0	144.4	54.7	102.0	30.3	273.6	181.1
GGT	IU/L	0-2	0.4	0.9	0.0	0.0	0.0	0.0	0.6	1.3
Total Bilirubin	mg/dl	0.0-0.3	0.2	0.0	0.2	0.0	0.2	0.0	0.2	0.1
Total Protein	g/dl	4.1-6.4	5.6	0.2	5.8	0.3	5.4	0.2	4.5	0.0
Albumin	g/dl	2.5-3.9	2.8	0.7	3.3	0.2	3.1	0.1	3.1	0.2
Blood Urea Nitrogen	mg/dl	14-32	14.0	4.1	17.4	1.8	15.4	1.1	17.0	1.2
Creatinine	mg/dl	0.1-0.6	0.2	0.1	0.2	0.0	0.2	0.0	0.2	0.1
Phosphorus	mg/dl	4.6-9.3	9.8	2.5	9.8	0.4	9.8	0.7	10.3	1.9
Calcium	mg/dl	7.6-10.7	9.7	0.2	9.9	0.1	9.9	0.3	9.8	0.2
Sodium	mEq/l	146-155	161.4	0.9	162.0	0.7	161.6	0.5	161.2	0.8
Chloride	mEq/l	103-115	115.0	0.0	115.0	0.0	115.0	0.0	115.0	0.0
Potassium	mEq/l	3.4-5.5	8.3	0.4	8.2	0.3	8.4	0.4	8.2	0.3
B/C Ratio		21-127	63.0	35.6	87.0	9.1	77.0	5.7	68.0	38.5
Na/K Ratio		26.7-42.1	19.4	1.0	16.3	7.8	19.2	0.9	19.7	0.7
T4, Total	µg/dL		3.6	1.7	5.4	0.8	4.5	1.0	4.0	1.7

Fig. S9. Toxicity evaluation of PU-H71 in mice. (*a*) PU-H71 does not induce toxicity in organs targeted by other Hsp90 inhibitors. B6D2F1 mice (13 males and 13 females) were administered vehicle or the indicated doses of PU-H71 3× week for 21 days. (*b*) Mice treated with PU-H71 suffered minor weight loss only at a dose exceeding with 25% the therapeutically required dose. B6D2F1 mice (13 males and 13 females) were administered vehicle or the indicated doses of PU-H71 3× week for 21 days. (*c*) PU-H71 does not induce hematologic, renal, liver, or thyroid toxicity in mice. B6D2F1 mice (13 males and 13 females) were administered Vehicle or the indicated doses of PU-H71 3× week for 21 days. Blood for hematology and clinical chemistry was collected on day 22 from 5 mice/group. Average values are presented.