Supporting Information

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SI Results

Currently, one of the major limitations in studying p97 function is the lack of a potent, selective, and reversible small-molecule inhibitor that has a defined mechanism of action. During the course of this work, EerI (1) and 2-anilino-4-aryl-1,3-thiazole (Myriad 12 and Myriad 19) (2) were reported to be p97 inhibitors. To understand how these compounds compare with DBeQ (N^2, N^4-dibenzylquinazoline-2,4-diamine), we evaluated their properties using our suite of assays (Table S2) (2). In our hands, the Myriad compounds were less potent p97 inhibitors both in vitro and in cells (IC_{50} of 15–40 μM) compared with what has been reported (3). We do not fully understand the basis for this discrepancy but would like to note that different in vitro assay methods were used (coupled enzyme assay vs. Biomol Green), and the cell-based assay used by Bursavich et al. (3) involved long exposure to compound and monitored accumulation of an Ub^{G76V}-luciferase reporter as opposed to degradation. A potential problem with the accumulation format was noted by Alvarez-Castelao et al. (4), who demonstrated that accumulation of three ubiquitin–proteasome system (UPS) reporters in response to proteasome inhibitors is driven in large measure by upregulation of the constructs' cytomegalovirus promoter. It is also worth noting that the Myriad compounds interfere with luciferase read-outs, which is in agreement with a recent report on a related scaffold (5). In addition, the Myriad compounds inhibited degradation of a p97-independent substrate by 48% at twofold above the IC_{50} at which they inhibited degradation of a p97-dependent substrate (Fig. S7). By comparison, the IC_{50}s of DBeQ toward p97-dependent and -independent substrates differed by 20-fold. We identified the same scaffold (compound **33**; Table S1) as the original Myriad hit in the 218K HTS, but subsequent assays revealed that this hit was of low potency, and it stabilized both the p97-dependent and -independent reporters. Thus, although we confirmed that the top Myriad compounds inhibit p97, they exhibited properties that may compromise their utility. By contrast, EerI proved to be a specific inhibitor of degradation of the p97-dependent reporter in our assays, but this compound exhibited other characteristics, including interference with luciferase read-outs and irreversibility, that circumscribe its use for some applications. Moreover, EerI has been reported to inhibit other cellular components, including ataxin 3 (1) and Sec61 (6). Notably, the mechanism by which EerI inhibits p97 is not understood. Interestingly, EerI did not inhibit ATPase activity of purified p97 (Table S2), and it has been suggested that it needs to be metabolized into an active compound to exert its inhibitory effect (1, 6). This could explain why high concentrations of EerI are needed to inhibit Sec61-mediated translocation in vitro (6).

SI Methods

Materials. Antibodies used in this study were anti-GFP (BD Biosciences), anti-luciferase (Promega), anti-p97 (Research Diagnostics), anti-LC3 (Medical Biological Lab), anti–β-actin (Cell Signaling Technology), anti-Histone H1.0 (Abcam), anti–β-tubulin (Sigma), anti-p21 (BD Transduction Laboratories), and anti-CHOP (Cell Signaling Technology). Chemicals used in this study were MG132 (Biomol), bafilomycin A1, and staurosporine (LC Laboratories), chloroquine and puromycin (Sigma), cycloheximide and Z-VAD(OMe)FMK (EMD Bioscience), and 1541 (ASINEX). Cells were grown on a 96-well cellstar black μclear bottom plate (ISC Bioexpress) for live-cell imaging on an automated microscope (ImageXpress Micro; Molecular Devices). FACS was carried out on an Accuri C6 Flow Cytometer (Accuri Cytometers). Luciferase intensity was determined on an Analyst AD plate reader (Molecular Devices). Plasmids for *Escherichia coli* recombinant protein expression and mammalian cell transfection, cell lines, and siRNAs used in this study are listed in Tables S3–S5. All buffers were prepared in deionized water and filtered through a 0.2-μm filter.

Recombinant Protein Expression and Purification. All buffers were prepared in deionized water and filtered through a 0.2 μm-filter. For recombinant expression of mouse p97, *E. coli* BL21 (DE3) containing the desired plasmid was grown in LB medium containing 50 μg/L ampicillin with shaking at 37 °C to an OD_{600} of 0.5. The cell culture was cooled down to 22 °C and induced with 1 mM isopropyl-beta-D-thiogalactopyranoside and harvested 8–10 h later by centrifugation. The cell pellet (≈6 g from 2 L) was suspended in 30 mL lysis buffer [100 mM Tris (pH 7.4), 500 mM KCl, 5 mM $MgCl_2$, 20 mM imidazole, 5% glycerol, 2 mM β-mercaptoethanol, and protease inhibitor tablet (Roche)]. The cells (held in an ice bath) were lysed by six 30-s pulses of sonication, separated by 2-min intervals. The lysate was centrifuged at 20,000 × *g* for 45 min at 4 °C, and the resulting supernatant was loaded onto a Ni-NTA column [5-mL suspension, preequilibrated with wash buffer (50 mL, 50 mM Hepes [pH 7.4], 150 mM KCl, 5 mM $MgCl_2$, and 20 mM imidazole)] and incubated at 4 °C with rotation for 30 min. The column was then flushed with wash buffer (100 mL), and His_6-tagged p97 was eluted by stepwise application of 10 mL of imidazole elution buffer (50 mM, 100 mM, 150 mM, 200 mM, or 250 mM imidazole in wash buffer). Fractions from the 200-mM and 250-mM imidazole steps were combined and concentrated with an Amicon Ultra-15 centrifugal filter unit (nominal molecular weight limit = 100 kDa). The mixture (0.5 mL of 20 mg/mL) was then fractionated with a gel filtration column (Tricorn Superdex 200; GE Healthcare), eluted with GF buffer [20 mM Hepes (pH 7.4), 250 mM KCl, and 1 mM $MgCl_2$] at 0.5 mL/min flow rate, and fractions corresponding to an apparent molecular weight of 500–600 kDa were collected and analyzed by 4–12% SDS/PAGE to evaluate purity (Invitrogen). Fractions that contained p97 of ≥95% purity were concentrated to 5 mg/mL, exchanged into storage buffer [20 mM Hepes (pH 7.4), 250 mM KCl, 1 mM $MgCl_2$, 5% glycerol, and 1 mM DTT], aliquoted, frozen in liquid nitrogen, and stored at −80 °C.

High-Throughput Screening (HTS) Assay Reagents. Kinase-Glo (Promega, part K1214) was purchased from Promega. Microtiter plates (1,536 wells) were purchased from Greiner. All chemicals were from Sigma-Aldrich unless otherwise stated. Tris was from Invitrogen. The Molecular Libraries Small Molecule Repository (MLSMR) library was provided by BioFocus DPI through the National Institutes of Health's Roadmap: Molecular Libraries Initiative. Details regarding compound selection for this library can be found online at http://mli.nih.gov/mli/compound-repository/mlsmr-compounds/. Briefly, the MLSMR library is a highly diversified collection of small molecules (more than 50% of compounds exhibit molecular weights between 350 and 410 g/mol) comprising both synthetic and natural products, from either commercial or academic sources, that can be grouped into the three following categories: (*i*) specialty sets of known bioactive compounds, such as drugs and toxins, (*ii*) focused libraries aimed at specific target classes, and (*iii*) diversity sets covering a large area of the chemical space.

HTS p97 Microtiter Plate Assay. HTS campaign experiments were executed on a GNF/Kalypsys HTS platform, using integrated liquid handlers, incubators, and a Viewlux multimode plate reader (Perkin-Elmer). Before the start of the assay 2.5 μL of Assay Buffer [1 mM DTT, 50 mM Tris HCl, 20 mM $MgCl_2$, and 1 mM EDTA (pH 8.0), filtered at 0.22 μm] was dispensed into columns 1 and 2 of 1,536-well assay plates. The remaining 46 columns were filled with 2.5 μL of Assay Buffer supplemented with 0.42 μM p97 protein. Next, 40 nL of test compounds (8 μM final nominal concentration) or DMSO alone (0.8% final concentration) was distributed into the appropriate wells. The plates were then incubated for 30 min at 25 °C. The assay was started by the addition of 2.5 μL of 10 mM Tris supplemented with 10 μM ATP to all wells. The plates were then incubated for 3 h at 25 °C. After incubation, 5 μL of Kinase Glo reagent was added to all 48 columns, and plates were incubated for another 10 min at 25 °C. Plates were centrifuged, and luminescence was measured by the ViewLux microplate reader.

HTS Assay Quality Control and Data Analysis. Raw data from the ViewLux plate reader were uploaded to an institutional HTS database (MDL Information Systems). Three quality control parameters were calculated during the screening on a per-plate basis: (*i*) the signal-to-basal ratio (S/B); (*ii*) the coefficient for variation [CV; CV = (SD/mean) × 100] for all compound test wells; and (*iii*) Z- or Z′-factor [Z′-factor = 1 – [[3 × (σp + σn)]/(μp – μn)], where σ is the SD and μ the mean for positive (p) and negative (n) controls] (7).

The percent inhibition for each compound was calculated using the following mathematical expression:

% Inhibition = [1-(Test_Compound – Median_High_Control)/(Median_Low_Control – Median_High_Control)] × 100

where "Test_Compound" is defined as wells containing test compound, "Low_Control" is defined as wells containing DMSO, and "High_Control" is defined as wells containing no p97 protein.

A mathematical algorithm was used to determine nominally inhibiting compounds in the primary screen (8). Two values were calculated: (*i*) the average percent inhibition of all compounds tested, and (*ii*) three times their SD. The sum of these two values was used as a cutoff parameter; that is, any compound that exhibited greater percent inhibition than the cutoff parameter (i.e., exhibiting inhibition >14.53%) was declared active. From the 217,968 MLSMR compounds tested in the primary screen, 923 compounds were declared to be active. Of the 923 active compounds, 759 were available from the MLSMR and ordered for retesting to confirm activity.

The confirmation assay was run in the exact same conditions as the primary HTS, except that the 759 compounds were tested in triplicate and activity values for each compound were reported as the average percentage activation of the three measurements, plus or minus the associated SD. Using the same cutoff parameter as that for the primary HTS, 333 compounds confirmed activity. Results of the HTS campaign are available from the PubChem database (http://www.ncbi.nlm.nih.gov/sites/entrez?db=pcassay&term=p97). Alternatively, the Assay Identifier number (AID) 1481 can be used to search for the primary p97 HTS screen results in PubChem and AID1517 for the confirmation assay results.

HTS Assay for PubChem AID1534. Before the start of the assay 18 μL of Assay Buffer [1 mM DTT, 50 mM Tris HCl, 20 mM $MgCl_2$, and 1 mM EDTA (pH 8.0), filtered at 0.22 μm] was dispensed into columns 1 and 2, rows O and P of 384-well assay plates. The remaining wells were filled with 18 μL of Assay Buffer supplemented with 0.42 μM p97 protein. Next, 50 nL of test compounds or DMSO alone (0.8% final concentration) were distributed into the appropriate wells. The plates were then incubated for 15 min at 25 °C. The assay was started by the addition of 1 μL of 10 mM Tris supplemented with 100 μM ATP to all wells. The plates were then incubated for 1 h at 25 °C. After incubation, 20 μL of Kinase Glo reagent was added to all 24 columns, and plates were incubated for another 10 min at 25 °C. Plates were centrifuged, and luminescence was measured by the Envision microplate reader. The percent inhibition for each compound was calculated using the mathematical expression described above.

For each test compound, percent inhibition was plotted against compound concentration. A four-parameter equation describing a sigmoidal dose–response curve was then fitted with adjustable baseline using Assay Explorer software (MDL Information Systems). The reported IC_{50} values were generated from fitted curves by solving for the X-intercept value at the 50% inhibition level of the Y-intercept value. In cases where the highest concentration tested (i.e., 50 μM) did not result in >50% inhibition, the IC_{50} was determined manually as >50 μM. Compounds with an IC_{50} >10 μM were considered inactive. Compounds with an IC_{50} ≤10 μM were considered active.

Any compound with a percent inhibition value <50% at all test concentrations was assigned an activity score of 0. Any compound with a percent inhibition value >50% at any test concentration was assigned an activity score greater than 0. Activity score was then ranked by the potency, with the most potent compounds assigned the highest activity scores. Activity scores for the active compounds of this assay have a range of 22–100, and all inactive compounds are assigned an activity score of 0.

Manual ATPase Assay. Assay Buffer [20 μL of 2.5× concentration, where 1× = 50 mM Tris (pH 7.4), 20 mM $MgCl_2$, 1 mM EDTA, and 0.5 mM tris(2-carboxyethyl)phosphine (TCEP)] was dispensed into each well of a 96-well plate. Purified p97 (25 μL of 50 μM) was diluted in 975 μL of 1× Assay Buffer, and 10 μL was dispensed in each well. Test compound (10 μL) or 5% DMSO (10 μL) was then added to each well, and the plate was incubated at room temperature for 10 min. The ATPase assay was carried out by adding to each well 10 μL of 500 μM ATP (pH 7.5), incubating at room temperature for 60 min, and then adding 50 μL Kinase Glo Plus reagent (Promega), followed by a final 10-min incubation at room temperature in the dark. Luminescence was read on an Analyst AD (Molecular Devices). Compounds were assayed at a range of concentrations (0, 0.048, 0.24, 1.2, 6, and 30 μM) in triplicate. The percent of remaining activity for each reaction was calculated using the following mathematical expression: [(Test Compound – High Control)/(Low Control – High Control)] × 100. Test_Compound is defined as wells containing test compound, Low_Control is defined as wells containing DMSO, High_Control is defined as wells containing no p97 protein. IC_{50} values were calculated from fitting the percentage of remaining activity (%RA) with various concentrations of compounds to a Langmuir equation [%RA = 100/(1 + [Compound]/IC_{50})] by nonlinear regression analysis using the JUMP IN program. The result was expressed as mean ± SE. For assaying with N-ethylmaleimide–sensitive factor (NSF) protein, reaction time was 1.5 h after addition of ATP.

For assays with EerI, Myriad 12, 19, and oxidized Myriad 12, 50 μL of Biomol Green reagent (Enzo Life Sciences) was added to each well instead of kinase Glo Plus (Promega), and absorbance at 630 nm was measured. This was done because these compounds interfered with luciferase activity. The Lineweaver-Burk plot of the competitive inhibition of p97 (50 nM) by DBeQ (0, 0.63, 2.5, and 10 μM) was carried out with various ATP concentrations (50–700 μM) in Assay Buffer containing 0.01% Triton X-100, and ATPase activity was determined by addition of Biomol Green reagent.

Reporter Degradation Assay. Two dual reporter stable HeLa cell lines were used and are described elsewhere (2). One expressed the UFD reporter Ub^{G76V}-GFP (9) and the oxygen-dependent degradation domain of HIF1α fused to luciferase (ODD-Luc)

(10) and the second expressed Ub^{G76V}-GFP and luciferase fused to the ubiquitin-independent degradation domain of ornithine decarboxylase (Luc-ODC) (10). Trypsinized cells were aliquoted into each well of a 96-well plate (5,000 cells/well) and grown for 16 h. Cells were treated with modified DMEM (without phenol red, folic acid, riboflavin, and vitamin B12) containing MG132 (4 μM) for 1 h and washed with prewarmed PBS (100 μL) twice. Modified DMEM containing FBS (2.5%), cycloheximide (30 μg/mL), and DMSO or a test compound (0–30 μM) was added into each well. Plates were imaged on the ImageXpress Micro at different time points. For the ODD-Luc or Luc-ODC turnover assay, four identical 96-well white solid-bottom plates were prepared. At each time point, one plate was taken out from the incubator and luciferase activity measure on Analyst AD.

Human 26S Proteasome Activity Assay. Inhibition of hydrolysis of the fluorogenic proteasome substrate (succinyl-Leu-Leu-Val-Tyr-AMC, 60 μM; Boston Biochem) by purified human 26S proteasome (1 nM) from Enzo Life Science in Assay Buffer containing ATP [50 mM Tris (pH 7.4), 20 mM $MgCl_2$, 1 mM EDTA, 0.5 mM TCEP, and 100 μM ATP]. Fluorescence intensity was monitored every 5 min over 60 min. IC_{50} values were calculated from fitting the %RA with various concentrations of compounds to a Langmuir equation [$\%RA = 100/(1 + [Compound]/IC_{50})$] by nonlinear regression analysis using the JUMP IN program. The result was expressed a mean ± SE.

Kinase Profiling. Pyrazolopyrimidine (ACJI-47; Table S2) is an adenine mimetic kinase inhibitor (11) and was synthesized according to the published procedure (12). The ATP acyl-phosphate probe was synthesized as described previously, except that desthiobiotin was substituted for biotin (13). Human HuH7 cell pellets were lysed in 25 mM Tris (pH 7.6), 150 mM NaCl, 1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 1% Tergitol Nonidet P-40 type, and 1% vol/vol phosphatase inhibitor mixture II (EMD/Calbiochem, #524625) with a tip sonicator and then cleared by centrifugation at 100,000 × *g* for 30 min. Cleared lysates were gel filtered (BioRad 10DG) into reaction buffer [20 mM Hepes (pH 7.8), 150 mM NaCl, 0.1% triton X-100, and 1% vol/vol phosphatase inhibitor mixture II] to remove endogenous nucleotides. After gel filtration, lysates were adjusted to a 5-mg/mL concentration, and $MnCl_2$ was added to a final concentration of 20 mM. Gel-filtered lysate was incubated with either DBeQ (15 μM), ACJI-47 (15 μM), or vehicle control for 15 min, followed by labeling with the ATP acyl-phosphate probe (5 μM) for 10 min. Labeled samples were denatured and reduced in 6 M urea, 10 mM DTT at 65 °C for 15 min, and then alkylated with 40 mM iodoacetamide at 37 °C for 30 min. After another gel-filtration step to remove excess probe and processing reagents, the samples were digested with trypsin (0.015 mg/mL) at 37 °C for 1 h. Desthiobiotinylated peptides were isolated on streptavidin resin and eluted in a 50% CH_3CN/water mixture containing 0.1% TFA for LC-MS/MS analysis.

Samples were analyzed on a Thermo LTQ ion trap mass spectrometer coupled with an Agilent 1100 series micro-HPLC system with autosampler essentially as previously described (14). The mass spectrometer was programmed to target *m/z* values of labeled protein kinase peptides previously found to be present in HuH7 lysates for MS/MS analysis. Quantitative signals for each labeled protein kinase peptide were derived by extraction of two to four known fragment ion signals from MS/MS spectra taken at the corresponding *m/z* values. The average aggregate fragment ion signal of duplicate inhibited samples was compared with the comparable signal from quadruplicate controls to give a percent inhibition value. A more detailed description of this method will be published shortly.

Western Blot Analysis. Cells were trypsinized and aliquoted (50,000 cells) into each well of a six-well plate and grown for ≈16 h. For the turnover assay, cells were first treated with MG132 (4 μM) for 1 h and washed with PBS (100 μL) twice. DMEM containing 2.5% FBS, cycloheximide (50 μg/mL), and the test compound was added into the well. Cells were harvested after a 2-h chase by trypsinization and centrifugation at 5000 × *g* for 4 min at 4 °C. Cell pellets were resuspended in ice-cold PBS (750 μL), then centrifuged. Cell pellets were immediately frozen in a −80 °C freezer and lysed in ice-cold Buffer A [50 mM Tris (pH 7.4), 1% Triton-X100, 200 mM NaCl, 1 mM EDTA, 2 mM $MgCl_2$, 5 mM TCEP, 10 mM NEM, protease inhibitor tablet (Roche), and Halt Phosphatase Inhibitor Mixture (Thermo Scientific)]. Soluble cell extract (100 μL for a six-well plate) was mixed with SDS-sample buffer (4×, 33 μL) and heated at 90 °C for 5 min. After centrifugation at 3,000 × *g* for 30 s, sample (25 or 50 μg) was loaded on a 4–12% SDS/PAGE. Proteins were transferred to nitroncellulose membranes and stained with Ponceau S. GFP-based reporters were detected with anti-GFP antibody, and luciferase-based reporters were detected with anti-luciferase antibody. Cell extract fractionation was carried out with a nuclear and cytoplasmic extraction reagent kit (Thermo Scientific), and the insoluble portion was prepared by dissolving the pellet in SDS-Urea buffer [4 M Urea, 5% SDS, 100 mM Tris (pH 7.5), 5 mM EDTA, 0.02% bromophenol blue, and 5 mM DTT].

Plasmid and siRNA Transfection. siRNA oligonucleotides purchased from Thermo Fisher Scientific or Qiagen were transfected into cells using Lipofectamine RNAiMAX (Invitrogen), and plasmids were transfected using FuGENE HD (Roche), according to the manufacturers' protocols.

Cellular Caspase Activity and Cellular Viability. Cells were seeded on a 384-well solid white plate (5,000 cells/well). Cells were transfected with luciferase siRNA or p97 siRNA (10 nM) for 48 h or treated with compounds for the indicated amount of time. Caspase-3/7 Glo, caspase-6 Glo, caspase-8 Glo, or caspase-9 Glo (Promega) was added into each well and mixed by shaking at 500 rpm for 1 min. Luminescence signal was determined after incubation at room temperature for 1 h. Cellular viability was determined with CellTiter-Glo reagent (Promega). To determine the IC_{50} of cellular viability, cells were treated with MG132 or DBeQ at seven concentrations (threefold serial dilutions starting at 33 μM) for 48 h. IC_{50} values were calculated from fitting the percentage of luminescence signal normalized to DMSO treated cells).

Purchased and Synthesized Compounds. The compounds identified as hits from the p97ATPase HTS of the National Institutes of Health Molecular Libraries Small Molecule Repository were purchased from Albany Molecular Research, Aldrich, Chembridge, ChemDiv, Interchim, Princeton Bio Molecular Research, and Ryan Scientific. The purchased compound samples were analyzed and purified, as necessary, using reverse-phase (RP) HPLC/UV/MS to >90% purity as measured by area under the curve at 214 nm. Myriad 12 and 19 were synthesized according to the published procedure (2). The oxidized version of Myriad 12 was prepared by oxidation of Myriad 12 using the 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) procedure. DBeQ was resynthesized as described previously (15). Compound structures were confirmed by high resolution mass spectrometry and ^1H NMR (Table S7).

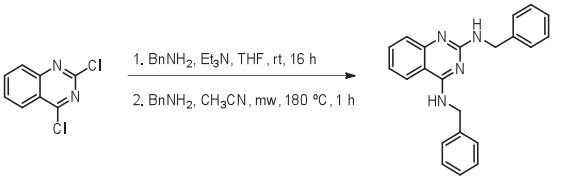
General Experimental Section for Small-Molecule Compounds. All reagents were used as received from the following suppliers: Ark Pharm, Aldrich Chemistry, and Fisher Scientific. Acetonitrile and THF were purified using the Innovative Technology PureSolv solvent purification system. The ^1H and ^{13}C spectra were recorded on a Bruker Avance 400 MHz spectrometer. Chemical shifts are reported in parts per million and were referenced to

residual proton solvent signals. Flash column chromatography separations were performed using the Teledyne Isco CombiFlash Rf using RediSep Rf silica gel columns. TLC was performed on Analtech UNIPLATE silica gel GHLF plates (gypsum inorganic hard layer with fluorescence). TLC plates were developed using iodine vapor. Automated preparative RP HPLC purification was performed using an Agilent 1200 Mass-Directed Fractionation system (Prep Pump G1361 with gradient extension, Make-up pump G1311A, pH modification pump G1311A, HTS PAL autosampler, UV-DAD detection G1315D, Fraction Collector G1364B, and Agilent 6120 quadrapole spectrometer G6120A). The preparative chromatography conditions included a Waters X-Bridge C18 column (19 × 150 mm, 5 um, with 19 × 10-mm guard column), elution with a water and acetonitrile gradient, which increases 20% in acetonitrile content over 4 min at a flow rate of 20 mL/min (modified to pH 9.8 through addition of NH_4OH by auxiliary pump), and sample dilution in DMSO. The preparative gradient, triggering thresholds, and UV wavelength were selected according to the analytical RP HPLC analysis of each crude sample. The analytical method used an Agilent 1200 RRLC system with UV detection (Agilent 1200 DAD SL) and mass detection (Agilent 6224 TOF). The analytical method conditions included a Waters Aquity BEH C18 column (2.1 × 50 mm, 1.7 um) and elution with a linear gradient of 5% acetonitrile in pH 9.8 buffered aqueous ammonium formate to 100% acetonitrile at 0.4 mL/min flow rate. Compound purity was measured on the basis of peak integration (area under the curve) from UV/vis absorbance (at 214 nm), and compound identity was determined on the basis of mass analysis. All compounds used for biological studies have purity >90% (Table S7).

Synthesis of Myriad 12, 19, and the Oxidized Version of Myriad 12 (KSC-16-5) (Scheme S1). Myriad 12 and 19 were synthesized according to the published procedure (2). The oxidized version of Myriad 12 was prepared by oxidation of Myriad 12 using the DDQ procedure.

4-(4-(4-chlorophenyl)thiazol-2-ylimino)cyclohexa-2,5-dienone (KSC-16-5). Myriad 12 [(4-(4-chlorophenyl)thiazol-2-ylamino)phenol); 122 mg (0.40 mmol)] was dissolved in 50 mL of dichloromethane. DDQ [183 mg (0.80 mmol)] was added. The mixture was stirred at room temperature for 16 h. The solvent was removed, and residue was purified by flash chromatography to give a dark-red solid (110 mg, 91%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.51–8.39 (m, 1H), 7.83–7.70 (m, 2H), 7.63 (s, 1H), 7.41–7.30 (m, 2H), 7.24–7.10 (m, 1H), 6.63 (ddd, J = 2.2, 6.3, 10.7 Hz, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 187.5, 169.8, 157.8, 154.8, 142.2, 134.8, 134.1, 133.2, 132.2, 130.9, 129.2, 127.6, 117.7. HRMS (*m/z*): calcd. for $C_{15}H_{10}ClN_2OS$ (M+H) 301.0202; found 301.0197.

Synthesized DBeQ (Scheme S2). ***N-benzyl-2-chloroquinazolin-4-amine.*** 2,4-dichloroquinazoline (2.4 g, 12.3 mmol) was suspended in 20 mL of THF. Triethylamine (2.1 mL, 14.7 mmol) was added, followed by the addition of benzylamine (1.4 mL, 12.9 mmol). The mixture was stirred at room temperature for 16 h. The mixture was diluted with EtOAc, filtered, and the filtrate was concentrated. The residue was purified by silica gel chromatography (EtOAc: hexanes = 1:3, Rf = 0.5) to give a colorless solid (1.6 g, 47%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.87–7.73 (m, 2H), 7.68 (d, J = 8.2 Hz, 1H), 7.54–7.32 (m, 6H), 6.10 (s, 1H), 4.90 (d, J = 5.3 Hz, 2H).



N^2,N^4-dibenzylquinazoline-2,4-diamine (DBeQ, KSC-16-67). To a suspension of *N*-benzyl-2-chloroquinazolin-4-amine (30 mg, 0.11 mmol) in CH_3CN (1 mL) was added benzylamine (0.025 mL, 0.22 mmol, 2 equiv.). The mixture was heated to 180 °C for 1 h under microwave irradiation. The solvent was removed under vacuum, the residue was suspended in EtOAc, washed with saturated $NaHCO_3$, and the layers were separated. The organic layer was dried over $MgSO_4$ and concentrated under vacuum. The residue was purified by silica gel chromatography (EtOAc, Rf = 0.7) to give a colorless solid (37 mg, 98%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.65–7.55 (m, 1H), 7.51 (d, J = 8.3 Hz, 2H), 7.45–7.22 (m, 10H), 7.10 (ddd, J = 1.3, 6.8, 8.1 Hz, 1H), 5.82 (s, 1H), 5.30 (s, 1H), 4.80 (d, J = 5.5 Hz, 2H), 4.76 (d, J = 5.8 Hz, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) 160.1, 159.6, 152.2, 140.1, 138.7, 132.8, 128.7, 128.5, 128.0, 127.6, 127.5, 127.0, 125.7, 121.1, 120.8, 111.1, 45.6, 45.1. HRMS (*m/z*): calcd for $C_{22}H_{21}N_4$ (M+H) 341.1766; found 341.1763.

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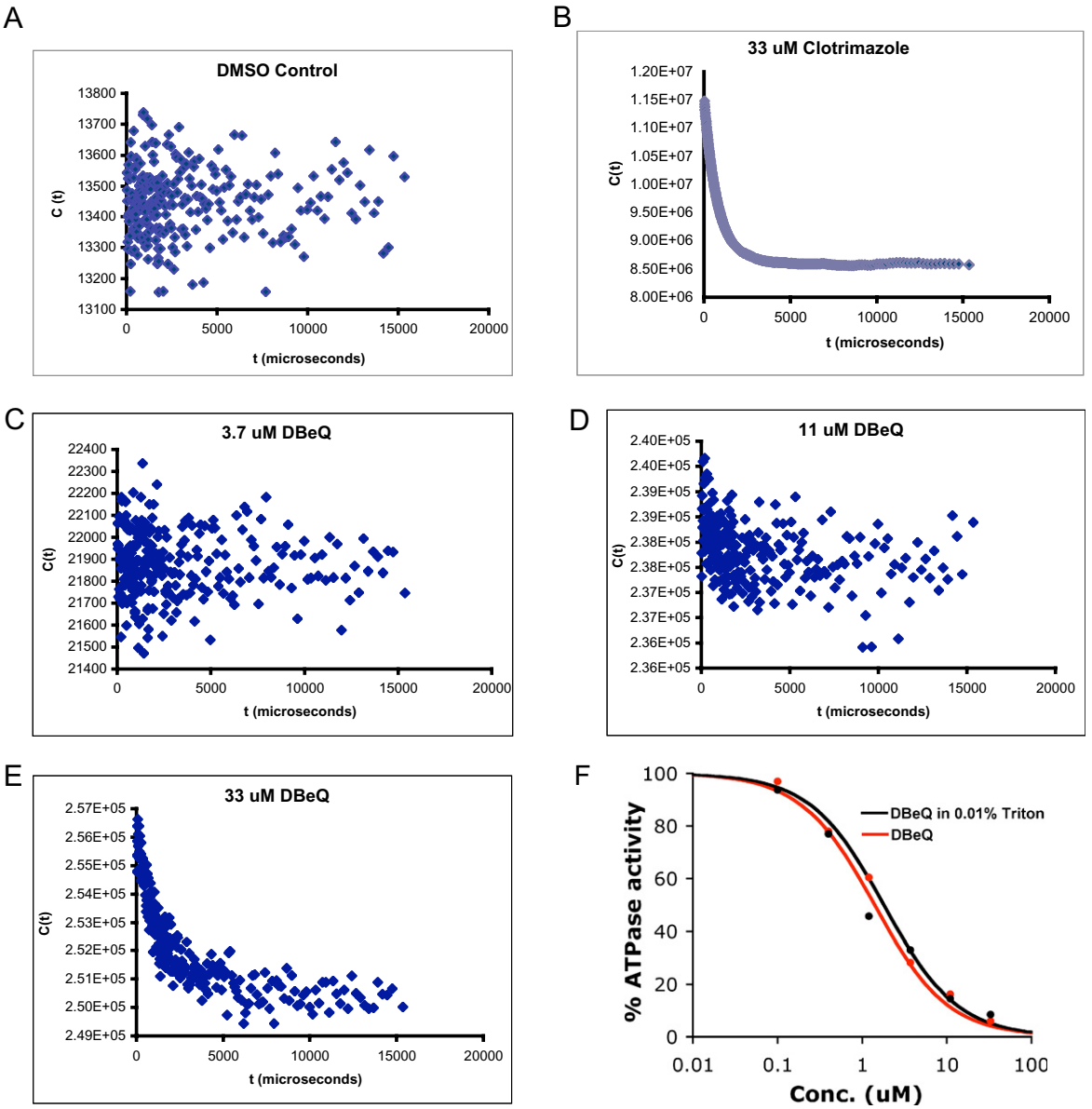


Fig. S1. DBeQ inhibits p97 ATPase activity independent of aggregate formation. Dynamic light scattering (DLS) was carried out on the Precision Detectors (PDDLS/CoolBatch 90T). Compounds were diluted to 1× ATPase assay buffer using the same protocol as for conducting ATPase assay. (*A*) DMSO, (*B*) clotrimazole used as positive control (1), (*C*) 3.7 μM DBeQ, (*D*) 11 μM DBeQ, and (*E*) 33 μM DBeQ. (*F*) Does–responsive curves of DBeQ on inhibiting ATPase activity in the presence (black line, 68 nM p97) or in the absence (red line, 250 nM p97) of 0.01% Triton X-100.

1. Seidler J, McGovern SL, Doman TN, Shoichet BK (2003) Identification and prediction of promiscuous aggregating inhibitors among known drugs. *J Med Chem* 46:4477–4486.

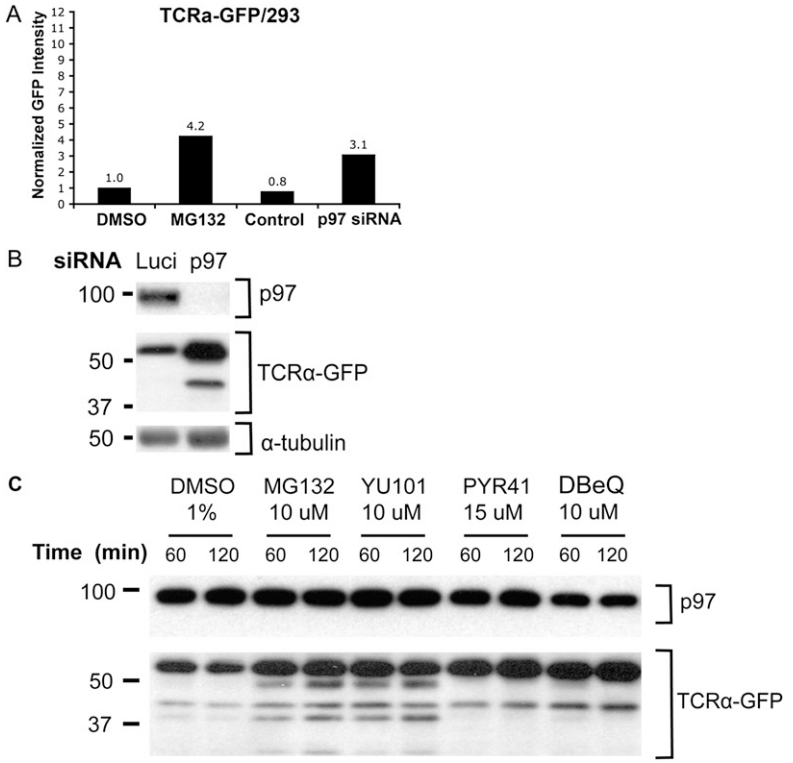


Fig. S2. Depletion of p97 stabilizes an endoplasmic reticulum-associated degradation (ERAD) reporter. TCRα-GFP (*A*) Hek293 cells stably expressing an ERAD reporter, TCRα-GFP, were assayed by FACS to quantify the degree of reporter accumulation after proteasome inhibition by MG132 (10 μM, 2 h) or p97 depletion by siRNA. (*B*) Immunoblot analysis of p97 levels and TCRα-GFP accumulation in 293 cells subjected to siRNA directed against luciferase (Luci) or p97. (*C*) Degradation of TCRα-GFP was used to determine the effect of DBeQ on the ERAD pathway and was carried out as described in Fig. 1*A* (main text).

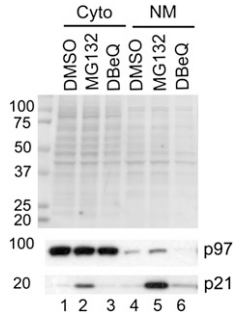


Fig. S3. Western blot to evaluate the protein level of p21. HeLa cells were incubated with 10 μM of MG132 or DBeQ for 5 h. The levels and distributions of p97 and p21 were determined by immunoblotting the cytosolic (Cyto) and nuclear plus membrane (NM) fractions.

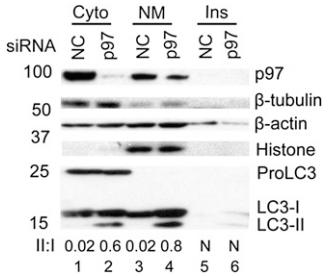


Fig. S4. Depletion of p97 stabilizes LC3-II. HeLa cells were transfected with negative control siRNA (NC) or p97 siRNA (10 nM) for 72 h, and the levels and distributions of the indicated proteins were determined by immunoblotting cytosolic (Cyto), nuclear plus membrane (NM), and insoluble (Ins) fractions.

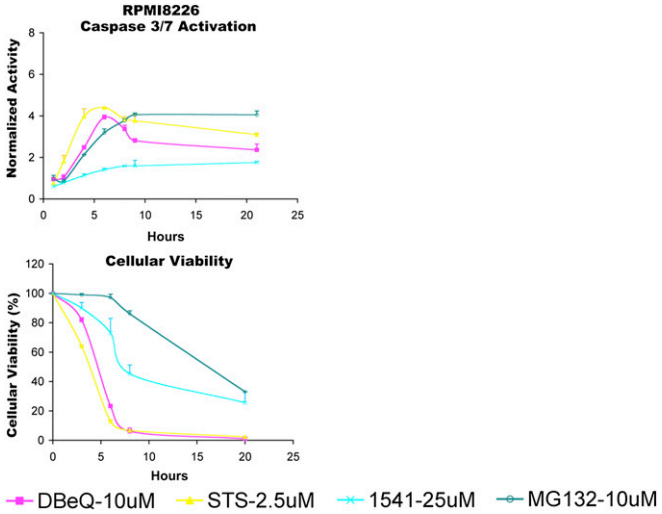


Fig. S5. Activation of caspases-3 and -7 and inhibition of cell growth by DBeQ. Time course of caspase-3/7 activity and cellular viability. *Upper:* RPMI8226 cells were incubated with the indicated concentrations of drugs for 1, 2, 4, 6, 8, 9, or 21 h before determination of caspases-3 plus -7 activities in total cell lysate. *Lower:* Cellular viability was determined using CellTiter-Glo after 3-, 6-, 8-, or 20-h incubation with drugs.

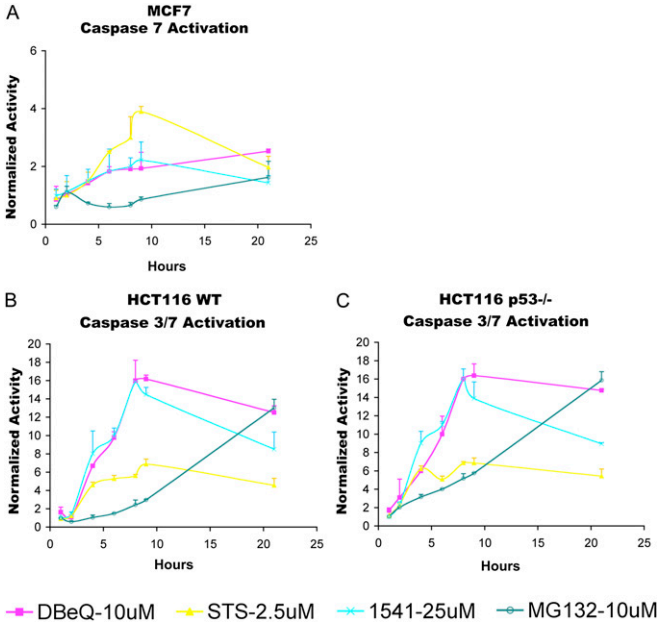


Fig. S6. Activation of caspases-3 and -7. (*A–C*) Same as Fig. S5, except MCF7, HCT116 WT, and HCT116 p53$^{-/-}$ cells were used, respectively.

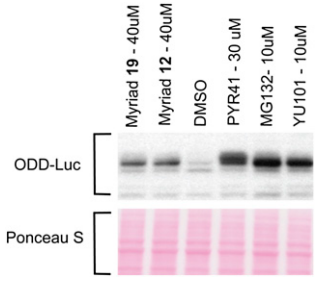


Fig. S7. Western blot to evaluate degradation of ODD-Luc by Myriad 12 and 19. HeLa cells stably expressing the Ub^{G76V}-GFP and ODD-Luc reporters were grown in six-well plates. Cells were treated with MG132 (4 μM for 1 h) to accumulate reporter, washed, and exposed to the indicated test compounds plus cycloheximide to initiate a chase. After 2 h, cells were harvested and evaluated by immunoblotting for the remaining ODD-Luc. The Ponceau S-stained filter serves as loading controls.

Table S1. Hits from two HTS screens for p97 inhibitors

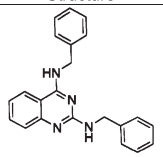
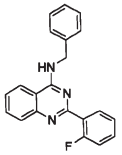
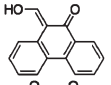
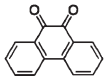
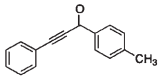
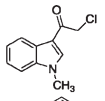
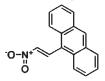
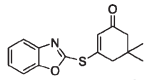
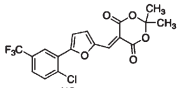
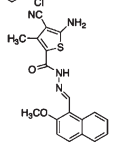
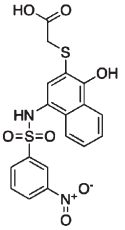
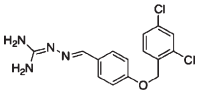
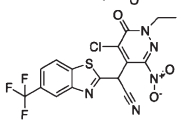
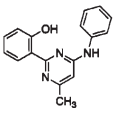
Compound ID†	Structure	Assay IC_{50} (μM)*		
		Ub^{G76V}-GFP degradation	ATPase activity (manual)	ATPase activity (HTS)
(**1**) CID676352 CID16472035		2.6 ± 0.3	1.5 ± 0.4	50
(**2**) KSC-1-150 CID886813		9.0 ± 1.3	3 ± 0.8	50
(**3**) JFD03665		1.1 ± 0.5	0.26 ± 0.05	>50
(**4**) KSC-1-127 CID6763		1.3 ± 0.5	3.5 ± 1.3	50
(**5**) JFD02342		3.4 ± 1.0	0.2 ± 0.02	47
(**6**) CID934321		3.6 ± 0.7	1.3 ± 0.2	50
(**7**) S09756		5.0 ± 1.0	0.8 ± 0.08	0.28
(**8**) JFD00597		6.6 ± 1.9	6.1 ± 0.8	>50
(**9**) SEW05182		7.5 ± 2.9	13 ± 1	10
(**10**) CID1109468		10 ± 2	2 ± 0.3	2.6
(**11**) KSC-1-132 CID2939678		8.4 ± 1	24 ± 11	50
(**12**) KSC-1-130 CID9586497		11 ± 3	24 ± 3	50
(**13**) KSC-1-204 CID3500086		13 ± 4	14 ± 5	50
(**14**) CID5413643		16 ± 3	10 ± 5	50

Table S1. Cont.

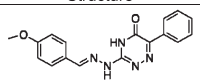
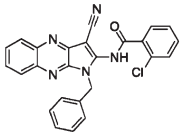
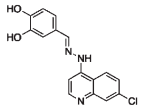
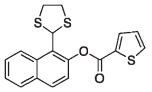
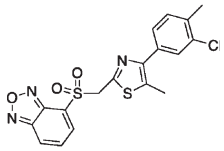
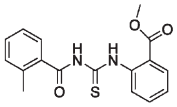
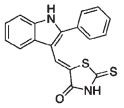
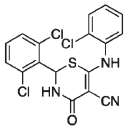
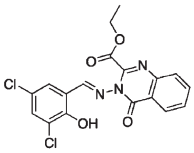
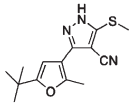
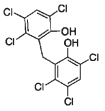
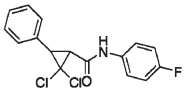
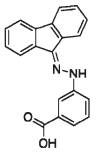
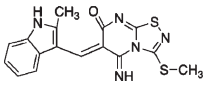
Compound ID*	Structure	Assay IC_{50} (μM)*		
		Ub^{G76V}-GFP degradation	ATPase activity (manual)	ATPase activity (HTS)
(**15**) KSC-1-131 CID2867539 CID9567210		16 ± 3	>30	50
(**16**) KSC-1-128 CID987585		17 ± 2	147 ± 58	50
(**17**) CID6524677		17 ± 5	4.9 ± 1.0	36
(**18**) SPB01017		19 ± 4	6.4 ± 2.3	>50
(**19**) HTS01888		19 ± 3	19 ± 5	14.5
(**20**) KSC-1-129 CID801956		21 ± 1.0	1.1 ± 0.18	50
(**21**) CID5756550		22 ± 3	34 ± 22	50
(**22**) CD07581		23 ± 4	4.0 ± 0.9	5.9
(**23**) CD02660		23 ± 7	>50	2.5
(**24**) SP01086		23 ± 8	>50	25
(**25**) CID3598		27 ± 10	2.7 ± 0.6	5.5
(**26**) CID2884885		27 ± 8	0.15 ± 0.05	50
(**27**) CID409928		36 ± 4	2.6 ± 0.5	50
(**28**) CID5875182		31 ± 4	8.3 ± 2.1	22

Table S1. Cont.

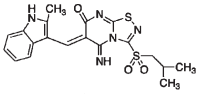
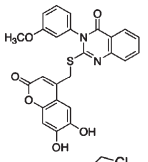
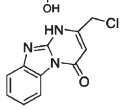
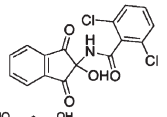
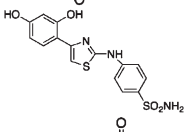
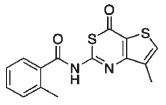
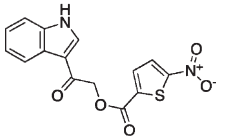
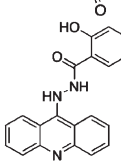
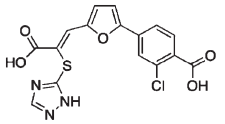
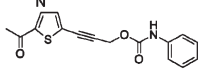
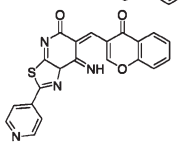
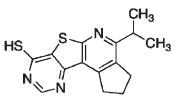
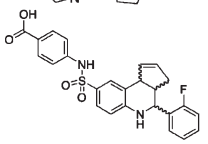
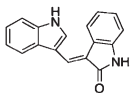
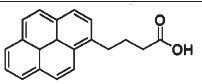
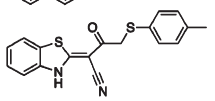
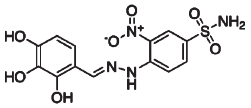
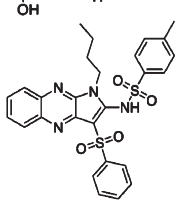
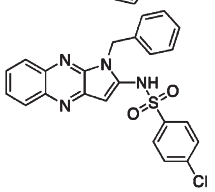
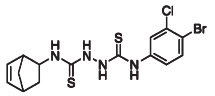
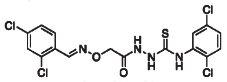
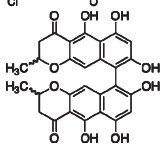
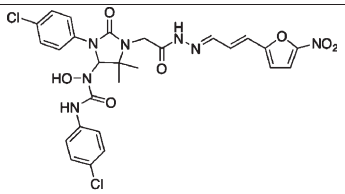
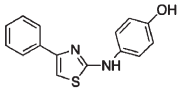
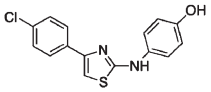
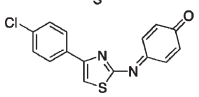
Compound ID†	Structure	Assay IC_{50} (μM)*		
		Ub^{G76V}-GFP degradation	ATPase activity (manual)	ATPase activity (HTS)
(**29**) CID:16194309		> 30	7.8 ± 1.9	12
(**30**) CID5691857		39 ± 10	4.1 ± 1	21
(**31**) KSC-1-206CID2384538		38 ± 4	3.9 ± 1.2	50
(**32**) KSC-1-202 CID2810445		42 ± 8	>30	50
(**33**) CID2175556		60 ± 24	3.7 ± 0.4	24
(**34**) KSC-1-201 CID2810155		62 ± 8	104 ± 47	50
(**35**) KSC-1-209 CID2472581		66 ± 8	92 ± 22	50
(**36**) KSC-1-199 CID4072379		68 ± 17	18 ± 3	50
(**37**) CID1625680		>30	0.85 ± 0.29	50
(**38**) KM08995		>30	1.4 ± 0.9	7.5
(**39**) CID1976446		>30	3.6 ± 1.7	11
(**40**) CID930238		>30	5.2 ± 1.8	42
(**41**) CID2978460		>30	9.0 ± 2.0	19
(**42**) CD00823		>30	9.2 ± 1.7	>50

Table S1. Cont.

Compound ID†	Structure	Assay IC_{50} (μM)*		
		Ub^{G76V}-GFP degradation	ATPase activity (manual)	ATPase activity (HTS)
(**43**) JFD01560		>30	12 ± 2	10
(**44**) KSC-1-133 CID 5559718		>30	8.8 ± 0.3	50
(**45**) KSC-1-205 CID5982653		>30	>30	50
(**46**) KSC-1-232 CID3787408		>30 Autofluorescent	>30	50
(**47**) KSC-1-233 CID2044030		>30 Autofluorescent	80 ± 37	50
(**48**) CD02506		>30	>30	3.2
(**49**) CD06082		>30	20	2.2
(**50**) CID160115		>30 Autofluorescent	>30	44

*Measurements were carried out in triplicate, and variance is expressed as the SE.
†Compounds with PubChem CID number are hits from the National Institutes of Health Molecular Libraries Small Molecule Repository. The remaining compounds are from the Maybridge HitFinder collection.

Table S2. Published chemical inhibitors of p97

Name	Structure	Assay IC_{50} (μM)*	
		Ub^{G76V}-GFP degradation	p97 ATPase
Eeyarestatin I (EerI)		3.7 ± 0.4	> 30
Myriad 19† KSC-1-275		26 ± 4	21 ± 2
Myriad 12† KSC-1-277		16 ± 2	20 ± 7
KSC-16-5‡		38 ± 8	68 ± 30

*Measurements were carried out in triplicate, and variance is expressed as the SD.
†Myriad 12 and 19 were readily oxidized to forms with similar activity as parent compounds.
‡Oxidized form of Myriad 12 was independently synthesized and tested.

Table S3. Plasmids used in this study

Plasmid no.	Plasmid name	Vector	Source and Reference
RDB2003	Murine p97-myc His/pcDNA4 TO	pcDNA4.1 TO (Invitrogen)	(1)
RDB2004	Murine p97 E578Q -myc His/pcDNA4 TO	pcDNA4.1 TO (Invitrogen)	(1)
RDB2120	Murine p97-pET28a	pET28a (Novagen)	(2)
RDB2122	Hamster NSF-pHisTrcHis-Flag-Myc	pHisTrcHis (Invitrogen)	Y. Ye*
RDB2402	C522A-p97-PET28a/DH5a	pET28a (Novagen)	(3)
RDB2405	QQmp97-myc His/pcDNA4.1TOsiRNA	pcDNA4.1 TO (Invitrogen)	(3)

*Provided by Y. Ye (National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD).

1. Dalal S, Rosser MF, Cyr DM, Hanson PI (2004) Distinct roles for the AAA ATPases NSF and p97 in the secretory pathway. *Mol Biol Cell* 15:637–648.
2. DeLaBarre B, Brunger AT (2003) Complete structure of p97/valosin-containing protein reveals communication between nucleotide domains. *Nat Struct Biol* 10:856–863.
3. Chou TF, Deshaies RJ (2011) Quantitative cell-based proteindegradation assays to identify and classify drugs that target the ubiquitin-proteasome system. *J Biol Chem*, in press.

Table S4. Cell lines used in this study

Cell line no.	Cell line name	Source and reference
DTC7	RPMI-8226	ATCC no. CCL-155
DTC12	TCRα-GFP/293	R. R. Kopito (1)
DTC22	HeLa	ATCC no. CCL 2
DTC23	Ub^{G76V}-GFP/Luc-ODC/HeLa	(2)
DTC25	Ub^{G76V}-GFP/ODD-Luc/HeLa	(2)
DTC44	HCT116 WT	B. Vogelstein (3)
DTC45	HCT116 $p53^{-/-}$	B. Vogelstein (3)
DTC63	MCF7	ATCC no. HTB-22
DTC65	Hek293	ATCC no. CRL1573
DTC69	MRC5	Coriell Institute AG-05965E

1. DeLaBarre B, Christianson JC, Kopito RR, Brunger AT (2006) Central pore residues mediate the p97/VCP activity required for ERAD. *Mol Cell* 22:451–462.
2. Chou TF, Deshaies RJ (2011) Quantitative cell-based proteindegradation assays to identify and classify drugs that target the ubiquitin-proteasome system. *J Biol Chem*, in press.
3. Bunz FD, et al. (1998) Requirement for p53 and p21 to sustain G2 arrest after DNA damage. *Science* 282:1497–1501.

Table S5. siRNAs used in this study

Target gene	Catalog no.
Luciferase	**Thermo Fisher Scientific** P-002099–01-20
p97/VCP	**Thermo Fisher Scientific** D008727060050
None	Negative control siRNA (**Qiagen** 1027310)

Table S6. Compound identifier, purity, and HRMS data for purchased and synthesized compounds

Cpd ID	KSC lot no.	PubChem CID	PubChem SID	Purity %	HRMS calcd.	HRMS found
1	KSC-16-67	676352	93575591	100.0	341.1766	341.1763
2	KSC-1-150	886813	87796231	100.0	330.1406	330.1408
3	NA					
4	KSC-1-127	6763	87326022	98.8	209.0602	209.0605
5	NA					
6	NA					
7	NA					
8	NA					
9	NA					
10	NA					
11	KSC-1-132	2939678	87334034		435.0320	Insufficient target
12	KSC-1-130	9586497	87334033	52.6 (partially hydrolyzed)*	337.0623	337.0615
13	KSC-1-204	3500086	87796244	100.0	444.0145	444.0133
14	NA					
15	KSC-1-131	9567210	87326025	97.4	322.1304	322.1296
16	KSC-1-128	987585	87326023	100.0	438.1121	438.1111
17	NA					
18	NA					
19	NA					
20	KSC-1-129	801956	87326024	97.9	329.0960	329.0955
21	NA					
22	NA					
23	NA					
24	NA					
25	NA					
26	NA					
27	NA					
28	NA					
29	NA					
30	NA					
31	KSC-1-206	2384538	87796246	100.0	234.0434	234.0429
32	KSC-1-202	2810445	87796242	Hydrolyzed*	349.9987	
33	NA					
34	KSC-1-201	2810155	87796241	100.0	317.0418	317.0411
35	KSC-1-209	2472581	92093138	94.2	331.0388	331.0388
36	KSC-1-199	4072379	87796243	95.7	346.1191	346.1187
37	NA					
38	NA					
39	NA					
40	NA					
41	NA					
42	NA					
43	NA					
44	KSC-1-133	5559718	87326026	95.6	339.0626	339.0617
45	KSC-1-205	5982653	87796245	Hydrolyzed*	369.0505	Insufficient target
46	KSC-1-232	3787408	92093144	97.0	535.1473	535.1466
47	KSC-1-233	2044030	92093145	97.8	449.0839	449.0829
48	NA					
49	NA					
50	NA					
Myriad 12	KSC-1-275	697280	92093146	91.4	269.0748	269.0744
Myriad 19	KSC-1-277	753704	92093147	90.2	303.0359	303.0343
Oxidized Myriad 12	KSC-16-5	45281169	92764872	95.1	301.0202	301.0197

Cpd, compound; KSC, University of Kansas Specialized Chemistry Center; CID, compound identifier; SID, substance accession identifier.
*Hydrolyzed: compound is not stable under basic LC conditions.

Other Supporting Information Files

Dataset S1 (XLS)