Adapted ATPase domain communication overcomes the cytotoxicity of p97 inhibitors

Yang Wei, Julia I. Toth, Gabrielle A. Blanco, Andrey A. Bobkov, and Matthew D. Petroski

From the NCI-designated Cancer Center, Sanford Burnham Prebys Medical Discovery Institute, La Jolla, California 92037

Edited by George N. DeMartino

The AAA⁺ ATPase p97 regulates ubiquitin-dependent protein homeostasis and has been pursued as a cancer drug target. The ATP-competitive inhibitor CB-5083 and allosteric inhibitor NMS-873 are the most advanced p97 inhibitors described to date. Previous studies have reported that their cytotoxicity can be readily overcome and involves single p97 mutations in the linker between the D1 and D2 ATPase domains and within D2. We report here that the proline 472 to leucine (P472L) mutation, in the D1–D2 linker and identified in CB-5083–resistant cells, desensitizes p97 to both inhibitor classes. This mutation does not disrupt the distinct D2-binding sites of the inhibitors. Instead, P472L changes ATPase domain communication within the p97 hexamer. P472L enhances cooperative D2 ATP binding and hydrolysis. This mechanism alters the function of the D1–D2 linker in the control of D2 activity involving the ATP-bound state of D1. Although increased D2 activity is sufficient to desensitize the P472L mutant to NMS-873, the mutant’s desensitization to CB-5083 also requires D1 ATPase domain function. Our study highlights the remarkable adaptability of p97 ATPase domain communication that enables escape from mechanistically distinct classes of cytotoxic p97 inhibitors.

This work was supported in part by a Padres Pedal the Cause Postdoctoral Fellowship (to Y. W.), National Institutes of Health Grants R01CA180150 and R01CA185300 (to M. D. P.), and Sanford Burnham Prebys NCI Cancer Center Support Grant P30 CA030199 from the National Institutes of Health. The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

1 To whom correspondence should be addressed: NCI-designated Cancer Center, Sanford Burnham Prebys Medical Discovery Institute, 10901 North Torrey Pines Rd., La Jolla, CA 92037. Tel.: 858-795-5167; E-mail: petroski@sbpdiscovery.org.

p97 (also known as valosin-containing protein and Cdc48 in yeast) is an essential AAA⁺ ATPase that functions as a protein segregase (1–3). The enzyme uses mechanical force generated by ATP-dependent conformational changes to extract ubiquitin-modified proteins from membranes, interacting proteins, and DNA. This function is critical for protein quality control to clear misfolded proteins by the proteasome (4). The over-reliance of cancers on this mechanism has led to the development of p97 inhibitors as candidate therapeutic agents (5, 6).

p97 functions as a hexamer where each subunit contains an N domain and two ATPase domains, D1 and D2, that are separated by short intervening linkers (7, 8). Each ATPase domain assembles into ring-like structures in the hexamer with the D1 ring stacked on top of the D2 ring surrounding a central pore. Structural and biochemical studies have identified distinct roles for D1 and D2 in substrate processing. ATP binding to D1 reorients the N domain upward to enable the binding of accessory proteins that deliver ubiquitinated substrates (9–12). D2 activity processes the substrate as it transits through the central pore (13). D1 ATP hydrolysis and partial ubiquitin removal by the N domain–bound deubiquitinating enzyme promote substrate release (13, 14).

Coordinated communication between the ATPase domains of p97 is necessary for its mechanism. Intra-subunit control of D1 and D2 activity is mediated by conformational changes involving the D1–D2 linker (9). Mutation of Walker A or B motifs found in both D1 and D2 that disrupt ATP binding or impair hydrolysis shows that ATP binding to one domain stimulates the other domain’s ATPase activity by increasing its affinity for ATP over ADP (15–18). D2 contributes most of the overall ATPase activity of p97 and, unlike D1, requires allosteric D2–D2 interactions for cooperative ATP hydrolysis (17, 19–22). This cooperativity involves arginine finger residues Arg-635 and Arg-638 from an adjacent D2 domain to catalyze γ-phosphosphate hydrolysis (15, 19–21, 23).

p97 mutations cause genetic disorders known as multisystem proteinopathy type-1 (MSP-1)² (24–26). These progressively degenerative diseases manifest in muscle, bone, and/or nervous system and are histologically identified by accumulated ubiquitin-modified proteins in afflicted tissues (26). The dominant, single p97 mutations found in MSP-1 patients are localized within the N and D1 region (25). Many MSP-1 mutants have increased D2 ATPase activity, decreased D1 ADP affinity, and aberrant N domain movement (11, 12, 27–34). A recent biochemical study found that an MSP-1 mutant unfolds a model ubiquitinated substrate faster than WT p97 (14). These findings suggest improperly coordinated ATPase activities of p97 may underlie MSP-1 pathogenesis.

p97 inhibitors developed as candidate cancer therapeutics provide new tools to understand the biological function of the enzyme. CB-5083 is an ATP-competitive and D2-selective inhibitor (35, 36). NMS-873 exclusively targets D2 activity but relies on an allosteric mechanism requiring D2 with ATP bound to inhibit cooperative D2 ATP hydrolysis (37). We and others have found that prolonged exposure of cancer cell lines

² The abbreviations used are: MSP-1, multisystem proteinopathy type-1; ITC, isothermal titration calorimetry; PDB, Protein Data Bank; PAM, protospacer adjacent motif; TR-FRET, time-resolved fluorescence energy transfer.
to cytotoxic concentrations of these inhibitors results in the emergence of resistant cells (35, 38, 39). The resistant cells harbor single amino acid mutations in D2 and the D1–D2 linker of p97 and are distinct from those found in MSP-1 patients (Fig. 1A). How the p97 mutants overcome the mechanism of action of CB-5083 or NMS-873 is unclear.

In this study, we biochemically analyzed p97 mutants identified from CB-5083–resistant and NMS-873–resistant HCT116 cells. We found that the CB-5083–resistant mutant harboring the proline 472 to leucine (P472L) mutation in the D1–D2 linker is also desensitized to NMS-873 but without disrupting the D2-binding sites of either inhibitor. The P472L mutation alleviates intra-subunit control of D2 activity by enhancing this domain’s cooperative ATP binding and hydrolysis. These findings suggest adapted ATPase domain communication circumvents the mechanisms of action of both ATP-competitive and allosteric p97 inhibitors to provide the enzyme’s essential function.

**Results**

**P472L mutation increases p97 ATPase activity and desensitizes the enzyme to its inhibitors**

We expressed and purified p97-harboring mutations identified from CB-5083–resistant (P472L, Q473P, G481A, N660K, or T688A) and NMS-873–resistant (A530T, R567H, or L639F) HCT116 cells (Fig. 1A) (35, 39). Mutants previously shown to disrupt the NMS-873 analog binding in vitro (K615V or N616F (37)), MSP-1 mutants harboring R95G or R155H (12, 26, 33,
The resulting cells were tested in cell viability dose-response experiments with CB-5083, NMS-873, and the proteasome inhibitor bortezomib using luminescent ATP detection. The parental HCT116 cells were used as a positive control, and NMS-873-resistant cells harboring the A530T p97 mutation were also included because they remain sensitive to CB-5083 (39). Although all the cells responded similarly to bortezomib based on resulting IC_{50} values, the P472L cells treated with either CB-5083 or NMS-873 and the A530T cells treated with NMS-873 did not (Fig. 2B). Thus, the P472L mutant overcomes the cytotoxicity of both classes of p97 inhibitors.

To measure cellular p97 activity, we generated HCT116 and P472L mutant cells that stably express the p97- and proteasome-dependent reporter ubiquitin–G76V–GFP (Ub–G76V–GFP). This constitutively ubiquitinated reporter is unfolded by p97 prior to its degradation by the proteasome (41, 42). p97 inhibition results in increased GFP fluorescence as a direct readout of perturbed ubiquitinated substrate unfolding (41).

Vehicle control (DMSO)-treated HCT116 and P472L mutant cells had similar steady-state levels of GFP fluorescence to indicate that the P472L mutant cells did not have increased substrate turnover (Fig. 2C). HCT116 cells demonstrated concentration-dependent reporter accumulation with CB-5083 and NMS-873. Consistent with the P472L mutant supporting p97 activity in the presence of both inhibitors, GFP fluorescence was largely unchanged with increasing concentrations of either inhibitor.

We performed Western blotting experiments to examine ubiquitin–protein conjugates, ATF4 and CHOP, that accumulate in response to p97 inhibition (35, 37). DMSO-treated P472L mutant cells showed unaffected ubiquitin–protein conjugates (Fig. 2D). CB-5083- and NMS-873–treated HCT116 cells and CB-5083–treated NMS-873–resistant A530T mutant cells had concentration-dependent increases in ubiquitin–protein conjugates ATF4 and CHOP. The P472L mutant cells did not appreciably accumulate these proteins in response to either inhibitor. These data indicate the P472L mutant does not overly alter ubiquitin-dependent protein homeostasis and is sufficient to desensitize cells to both classes of p97 inhibitors.

### P472L mutation does not disrupt the binding sites of p97 inhibitors

On-target mutations can disrupt inhibitor-binding sites to cause resistance to selective cytotoxic molecules (43). Because Pro-472 is in the D1–D2 linker and makes direct contact with an α-helix in D2 (see Fig. 1A), the leucine mutation could cause structural rearrangements in D2 that disrupt both CB-5083- and NMS-873–binding sites. To test this possibility, we performed biophysical experiments to compare the binding affinities of CB-5083 or NMS-873 to the P472L mutant and p97.

We used isothermal titration calorimetry (ITC) to measure the binding of CB-5083 to the D2 ATP-binding pockets of p97 and the P472L mutant. In experiments titrating CB-5083 on p97 and the P472L mutant (Fig. 3A), we obtained similar dissociation constants (K_{d} values) for CB-5083. Thus, the P472L mutant maintains an intact CB-5083–binding site.

To measure allosteric NMS-873 binding (37), we used two different approaches. p97 with bound BODIPY-FL–ATP and a
Adapted ATPase domain communication by the p97 P472L mutant

A

HCT116

Glu Val Pro\textsuperscript{472} Gin

G A G T G C C A C A G

PAM

P472L

Glu Val Leu\textsuperscript{472} Gin

G A A G T C T A C A G

PAM

B

HCT116: 335 ± 24 nM
A530T: 269 ± 17 nM
P472L: ND

HCT116: 2.2 ± 0.1 μM
A530T: ND
P472L: ND

HCT116: 4.4 ± 0.3 nM
A530T: 3.6 ± 0.6 nM
P472L: 5.2 ± 0.3 nM

% viability

[CB-5083], μM

0 1 10

% viability

[NMS-873], μM

0 1 10

% viability

[bortezomib], nM

0 100

C

integrated intensity

HCT116 P472L

integrated intensity

HCT116 P472L

integrated intensity

[CB-5083]

0 -

[CB-5083]

0 -

[CB-5083]

0 -

[NMS-873]

0 -

[NMS-873]

0 -

[NMS-873]

D

Ubiquitin

p97

ATF4

CHOP

HCT116 P472L A530T

HCT116 P472L A530T

HCT116 P472L A530T

HCT116 P472L A530T

HCT116 P472L A530T

HCT116 P472L A530T
terbium-labeled antibody that binds the N-terminal hexahistidine tag of recombinant p97 generates TR-FRET (39). Increasing concentrations of NMS-873 cause increased TR-FRET to indicate that the inhibitor enhances ATP binding to p97. This enables measurement of the apparent dissociation constant (K_D) of NMS-873 to ATP-bound p97. Increasing concentrations of NMS-873 added to the ATP-bound P472L mutant caused increasing TR-FRET (Fig. 3B). The resulting apparent dissociation constant was only modestly increased compared with that obtained for ATP-bound p97. We also measured NMS-873 binding to p97 with bound EDA–ADP–ATTO-495 by fluorescence polarization (Fig. 3C). Previous work demonstrated that ADP binding to p97 as measured by anisotropy is increased by NMS-873 in a concentration-dependent manner (37). We obtained similar dissociation constants for NMS-873 with respect to ADP-bound p97 and the P472L mutant. Moreover, NMS-873 had higher affinity to both proteins in the presence of bound ATP over ADP, reflecting its allosteric mechanism of action (37).

Figure 2. p97 P472L mutant cells are resistant to ATP-competitive and allosteric p97 inhibitors. A, P472L mutation was introduced into the p97 gene using homology-directed repair with CRISPR in HCT116 cells. Sequencing chromatograms from genomic DNA samples show incorporation of the missense C/T mutation to alter the Pro-472 codon and a silent A/G mutation that disrupts the targeted PAM in transfected and CB-5083–selected cell populations. B, P472L-edited HCT116 cell populations, control HCT116 cells, and NMS-873–resistant HCT116 cells harboring the A530T p97 mutation were tested in cell viability experiments. Inhibitors (2-fold serial dilutions from 5 μM CB-5083 or 20 μM NMS-873; 3-fold dilutions from 200 nM bortezomib) were added for 72 h (CB-5083 and NMS-873) or 48 h (bortezomib) prior to measuring ATP by luminescent detection. Resulting measurements were normalized to the maximum luminescence signal set at 100%. Data points represent the mean (n = 3) with S.D. IC_50 values with standard error (S.E.) from treatments that resulted in valid dose-response curves are indicated (ND, not determined). C, HCT116 and p97 P472L mutant cells were stably transfected with the p97 and proteasome substrate Ub–G76V–GFP and seeded to 96-well plates prior to incubating with CB-5083 (DMSO, 0.123, 0.37, and 1.1 μM) and NMS-873 (DMSO, 0.37, 1.1, and 3.3 μM) for 8 h. Integrated intensities (individual data points, mean, and, S.D. error) are shown. D, protein extracts from HCT116 and P472L cells treated with vehicle (DMSO) or inhibitors (0.3 or 2.5 μM, CB-5083; 2 or 5 μM, NMS-873) for 6 h were analyzed by Western blotting for ubiquitin, p97, ATF4, and CHOP.
Adapted ATPase domain communication by the p97 P472L mutant

Because P472L does not disrupt the binding sites of CB-5083 or NMS-873, the increased ATPase activity of the mutant could cause its desensitization to the inhibitors. To evaluate this, we first sought to determine whether the mutant’s increased activity is attributable to the D1 and/or D2 ATPase domains. We expressed and purified p97 and the P472L mutant with Walker A or Walker B motif mutations in D1 or D2 (Fig. 4A) (8, 16, 17, 44). A Walker A mutation (K251A in D1 and K524A in D2) disrupts ATP binding to the mutated domain to eliminate intra-subunit communication necessary for controlling the ATPase activity of the other domain. A Walker B mutation (E305Q in D1 and E578Q in D2) allows ATP binding to the mutated domain but impairs its hydrolysis. This type of mutation supports intra-subunit communication whereby ATP bound to the mutated domain stimulates the activity of the other domain through the bidirectional function of the D1–D2 linker (9, 16).

After determining enzyme concentrations that resulted in linear rates of ATP hydrolysis using colorimetric phosphate detection, ATP titration experiments were performed to obtain steady-state kinetic parameters of the enzymes (Fig. 4B and Table 2). The Walker motif mutations in D2 resulted in similar D1 catalytic efficiencies for the P472L mutant and p97 where both responded to the ATP-bound status of D2. Although these Walker motif mutations in D1 of p97 demonstrated ATP-dependent stimulation of D2 activity, D2 of the P472L mutant maintained elevated activity that modestly increased with ATP-bound D1.

We next evaluated whether the resistance of the P472L mutant to CB-5083 and NMS-873 relies exclusively on its increased D2 activity or whether altered intra-subunit communication between D1 and D2 is also involved. We measured ATPase activity inhibition of the P472L mutant and p97 proteins containing individual Walker motif mutations in D1 or D2 with increasing concentrations of CB-5083 (C) and NMS-873 (D) (n = 4, S.D. error). Resulting kinetic properties and IC50 values are summarized in Table 2.

Figure 4. P472L mutation increases D2 ATPase activity and alters its intra-subunit control by D1. A, schematic shows the location of the P472L mutation relative to Walker A and Walker B mutations in D1 and D2. B, ATP titration experiments (n = 4, S.D. error) were performed to obtain steady-state kinetic parameters of p97 and the P472L mutant proteins containing the indicated Walker motif mutations. The sensitivities of the ATPase activities of p97 and the P472L mutant with Walker motif mutations in D1 or D2 were evaluated in the presence of increasing concentrations of CB-5083 (C) and NMS-873 (D) (n = 4, S.D. error). Resulting kinetic properties and IC50 values are summarized in Table 2.

P472L mutation increases D2 ATPase activity and alters D1–D2 communication

20174 J. Biol. Chem. (2018) 293(52) 20169–20180

20174 J. Biol. Chem. (2018) 293(52) 20169–20180

20174 J. Biol. Chem. (2018) 293(52) 20169–20180

20174 J. Biol. Chem. (2018) 293(52) 20169–20180
Adapted ATPase domain communication by the p97 P472L mutant

Table 2
Kinetic properties and IC₅₀ values of CB-5083 and NMS-873 for p97 and the P472L mutant with D1 or D2 Walker motif mutations

|        | kₜₐ | kₐₑ | IC₅₀ (nm) |
|--------|------|------|----------|
| WT     | 91 ± 25 | 114 ± 0.9 | 125 ± 35 | 5 ± 0.5 | 14 ± 1 |
| D1     | 43 ± 5 | 0.27 ± 0.01 | 62 ± 0.7 | 43 ± 6 | 32 ± 4 |
| D1     | 66 ± 8 | 129 ± 0.4 | 187 ± 23 | 10 ± 1 | 16 ± 2 |
| D2     | 115 ± 14 | 0.25 ± 0.01 | 2.2 ± 0.3 | ND³ | ND |
| D2     | 37 ± 4 | 2.37 ± 0.07 | 64 ± 7 | 20 ± 5 | 513 ± 101 |

* Activity inhibition was not observed over the concentration range tested.

Table 2a
ATPase domain communication desensitizes the AAA⁺ ATPase domain communication by the p97 P472L mutant

|        | kₜₐ | kₐₑ | IC₅₀ (nm) |
|--------|------|------|----------|
| WT     | 91 ± 25 | 114 ± 0.9 | 125 ± 35 | 5 ± 0.5 | 14 ± 1 |
| D1     | 43 ± 5 | 0.27 ± 0.01 | 62 ± 0.7 | 43 ± 6 | 32 ± 4 |
| D1     | 66 ± 8 | 129 ± 0.4 | 187 ± 23 | 10 ± 1 | 16 ± 2 |
| D2     | 115 ± 14 | 0.25 ± 0.01 | 2.2 ± 0.3 | ND³ | ND |
| D2     | 37 ± 4 | 2.37 ± 0.07 | 64 ± 7 | 20 ± 5 | 513 ± 101 |

* ND means not determined.

Discussion

In this study, we have provided evidence that adapted ATPase domain communication desensitizes the AAA⁺ ATPase domain communication by the p97 P472L mutant to its current small molecule inhibitors. Through a biochemical analysis of p97 mutants identified in cells resistant to ATP-competitive or allosteric inhibitors, we determined that the P472L mutation, localized within the linker between its D1 and D2 ATPase domains, desensitizes the enzyme to both inhibitor classes. The underlying mechanism does not rely on disrupting the distinct D2-binding sites of the inhibitors. Our data suggest P472L alleviates intra-subunit control of D2 by D1 through enhanced cooperative D2 activity (Fig. 6). These
changes circumvent the distinct mechanisms of action of CB-5083 and NMS-873 necessary for productive p97 inhibition and support the enzyme’s essential function.

P472L alters the bidirectional role of the D1–D2 linker in intra-subunit communication. For p97, ATP binding to either D1 or D2 controls the other domain’s ATPase activity (16). This mechanism involves conformational changes mediated by the D1–D2 linker to increase ATP affinity over ADP (9, 15). The P472L mutant has similar D1 activity to p97 that is stimulated by ATP binding to D2, indicating that this function of the linker is unchanged. By contrast, the elevated D2 activity of the mutant is markedly less responsive to ATP-bound D1. This
Adapted ATPase domain communication by the p97 P472L mutant

Table 3
D2 nucleotide binding, kinetic properties, and IC50 values of CB-5083 and NMS-873 for p97 and the P472L mutant with R635A or R638A arginine finger mutations

|            | Kd, ADP | Kd, ATP | Km | kcat | kcat/Km | CB-5083 | NMS-873 |
|------------|---------|---------|-----|------|---------|---------|---------|
| WT         | 0.6 ± 0.07 | 1.8 ± 0.2 | 77 ± 9 | 8.6 ± 0.3 | 112 ± 14 | 8.1 ± 0.8 | 12 ± 1 |
| R635A      | 0.8 ± 0.1 | 4.6 ± 0.6 | 88 ± 11 | 0.29 ± 0.01 | 3.2 ± 0.4 | >8000* | >8000* |
| R638A      | 1.2 ± 0.1 | 3.5 ± 0.4 | 48 ± 4 | 0.39 ± 0.01 | 8.1 ± 0.7 | >8000* | >8000* |
| P472L      | 1.1 ± 0.1 | 0.4 ± 0.06 | 40 ± 5 | 37 ± 1 | 924 ± 114 | 118 ± 17 | 674 ± 58 |
| R635A      | 0.9 ± 0.1 | 2.6 ± 0.2 | 29 ± 5 | 0.94 ± 0.04 | 32 ± 5 | 31 ± 4 | 72 ± 12 |
| R638A      | 1.4 ± 0.1 | 4.0 ± 0.4 | 36 ± 3 | 1.14 ± 0.02 | 31 ± 3 | 79 ± 18 | 254 ± 29 |

* Activity inhibition was not observed over the concentration range tested.

Figure 6. Proposed desensitization mechanism of p97 to its ATP-competitive and allosteric inhibitors. NMS-873 allosterically inhibits p97 activity by blocking cooperative D2 ATP hydrolysis. CB-5083 functions as an ATP-competitive and D2-selective inhibitor. Our data suggest that intra-subunit control of D2 activity through ATP-bound D1 is critical for p97 sensitivity to this molecule (orange arrow). The P472L mutant, identified from CBB-5083-resistant cells, is desensitized to both inhibitors. This mechanism involves enhanced cooperative ATP binding and hydrolysis by D2 that alter its intra-subunit control by ATP-bound D1.

CB-5083 functions as a D2-selective and ATP-competitive p97 inhibitor (35, 36). Our data demonstrate, however, that disrupted ATP binding to D1 desensitizes p97 to the compound. This unexpected finding suggests intra-subunit communication of the ATP-bound status of D1 to D2 is critical for CB-5083 to optimally inhibit p97. We propose that the P472L mutant is desensitized to CB-5083 by overcoming this mechanism through increased cooperative D2 ATP binding and hydrolysis. This model is supported by our findings that impaired D2 activity and disrupted intra-subunit communication both sensitize the mutant to CB-5083.

Collectively, our study establishes that adapted ATPase domain communication can desensitize p97 to its current ATP-competitive and allosteric inhibitors. The underlying mechanism reveals unanticipated plasticity of the enzyme to provide its essential biological function. Unlike MSP-1 disease-causing mutants that also have elevated D2 ATPase activities (11, 12, 28, 32–34), the P472L mutant does not appear to generally impact ubiquitin-dependent protein homeostasis in cells. Considering that p97 function involves distinct roles attributed to D1 and D2 ATPases (13), we propose that the mutant has sufficiently controlled D1 activity for substrate recruitment and/or release to prevent aberrant processing through its increased D2 activity.

Experimental procedures

**p97 inhibitors**

NMS-873 was purchased from XcessBio and Sigma. CB-5083 was purchased from Selleckchem. Dry powders were stored at room temperature in a humidity-controlled desiccator with 10 mM liquid stocks prepared in 100% DMSO and stored at −80 °C. Compound identity and purity were assessed by LC-MS.

**Recombinant DNA, protein expression, and purification**

Baculovirus expression and purification of p97 were as described (39, 45). The p97 reporter Ub−G76V−GFP was generated as described (41, 42). Site-directed mutagenesis used QuickChange (Agilent). Because of low expression in insect cells, D2 arginine finger mutants and the K251A mutants were expressed from pET11b using Rosetta 2(DE3) bacteria (Novagen). Cultures were grown to an OD600 of 0.8 and induced with 0.8 mM isopropyl 1-thio-β-D-galactopyranoside for 4 h at 30 °C, and proteins were purified by nickel-nitrilotriacetic acid chromatography as for baculovirus-expressed p97. Protein concentrations were determined by Coomassie-stained SDS-poly-
acrylamide gel band intensity with BSA as a standard using a Licor Odyssey scanner and Image Studio software.

Cell culture, gene editing, and inhibitor profiling

HCT116 cells were purchased from ATCC, tested for mycoplasma, and authenticated before and at several points throughout the study. Sf9 and Hi5 insect cells were purchased from Thermofisher Scientific. HCT116 cell lines resistant to CB-5083 or NMS-873 were isolated and characterized as described and used 0.5 μM CB-5083 or 2 μM NMS-873 (39). Single amino acid p97 mutations identified during this study are indicated in bold in Fig. 1A. To generate engineered P472L cell lines, HCT116 cells were co-transfected with a Cas9-expressing plasmid (PX458, Addgene plasmid no. 48138) containing an sgRNA corresponding to bases 35,060,894 to 35,060,875 of the p97 gene exon 12 on chromosome 9 and a linear repair template corresponding to 35,060,974 to 35,060,787 with mutations at nucleotide 35,060,873 (G to A; disrupts PAM) and 35,060,868 (C to T; mutates Pro-472 codon to Leu). Exact sequences of the sgRNA and repair template DNA will be provided upon request. For cell viability experiments evaluating CB-5083, NMS-873, and bortezomib (Fig. 2B), transfected and CB-5083–selected (1.5 μM) H9262 cell populations were used. Sequence-verified P472L mutant cell lines isolated by limited dilution cloning without CB-5083 selection were used for Ub–G76V–GFP and Western blotting experiments.

Cells (HCT116, NMS-873–resistant p97 A530T (39), and engineered p97 P472L) were seeded to 96-well plates (5000 cells per well). After 24 h, 2-fold serial dilutions of CB-5083 (maximum 20 μM), NMS-873 (maximum 20 μM), and bortezomib (maximum 200 nM) were added, followed by an additional 48-h dilution cloning without CB-5083 selection were used for Ub–G76V–GFP and Western blotting experiments.

For p97 substrate reporter experiments, HCT116 and P472L cells were transfected with a plasmid encoding Ub–G76V–GFP and selected using 600 μM CB-5083 or NMS-873 were used for 6-h CB-5083 or NMS-873 treatments followed by an additional 48-h incubation. Cell viability was measured using Cell Titer-Glo (Promega). Resulting inhibition curves were fit in GraphPad Prism 7 to obtain IC50 values with standard error.

Ligand binding

ITC measurements were performed on a MicroCal iTC200 using 25 mM HEPES, pH 7.6, 1 mM MgCl2, 100 mM NaCl, and 0.5 mM tris(2-carboxyethyl)phosphine at 23 °C with 32 μM p97 or P472L mutant. Each titration used 19 injections of 2 μL of 300 μM CB-5083. Resulting data were fit using Origin software (Malvern) with a one-site binding model to obtain KD values.

TR-FRET assays were performed as described and used 36 μM p97 or P472L mutant proteins, 1 nM terbium-labeled anti-His antibody, and 50 nM BODIPY–FL–ATP with 2-fold serial dilutions of NMS-873 to 8 μM (39). After 1 h at room temperature, signals were recorded on an Envision microplate reader with excitation at 385 nm and emission at 485 and 520 nm. The emission ratio (520:485) data were fit to a hyperbolic equation in GraphPad Prism 7 to obtain apparent KD (EC50) values with respect to NMS-873.

Fluorescence polarization assays used the same buffer as ITC experiments supplemented with 0.05% Tween 20 and performed at room temperature. These experiments used p97 or P472L mutant proteins where the D1 ATPase has the Walker A mutation (K251A) and D2 has the Walker B mutation (E578Q) to attribute nucleotide binding to D2 without ATP hydrolysis. The 2-fold serial dilutions of p97 proteins (maximum 40 μM, purified from bacteria) were mixed with 20 nM BODIPY–FL–ATP (ThermoFisher Scientific) or 10 nM EDA–ADP–ATTO-495 (Jena Bioscience) in black low volume 384-well plates. After 30 min, fluorescence anisotropy was recorded on an Envision microplate reader (PerkinElmer Life Sciences) with excitation at 485 nm and emission at 595 nm. Background fluorescence (proteins without added fluorophores) was measured and subtracted from parallel and perpendicular intensities prior to anisotropy calculations. Data were fit in GraphPad Prism 7 using a ligand-binding quadratic equation to obtain KD values.

Author contributions—Y. W., J. I. T., and M. D. P. conceptualization; Y. W., J. I. T., G. A. B., A. A. B., and M. D. P. resources; Y. W., J. I. T., G. A. B., A. A. B., and M. D. P. investigation; Y. W., J. I. T., G. A. B., A. A. B., and M. D. P. writing-review and editing; M. D. P. supervision; M. D. P. funding acquisition; M. D. P. writing-original draft.

Acknowledgments—We thank Eduard Sergienko and Anthony Pinkerton for technical advice and assistance, Dieter Wolf for invaluable input on the manuscript, and Dorit Hanein, Niels Volkman, Robert Liddington, and Guy Salvesen for helpful discussions. PX458 was a gift from Feng Zhang (Addgene plasmid no. 48138).
Adapted ATPase domain communication by the p97 P472L mutant

References

1. Stach, L., and Freemont, P. S. (2017) The AAA⁺ ATPase p97, a cellular multitool. Biochem. J. 474, 2953–2976 CrossRef Medline
2. van den Boom, J., and Meyer, H. (2018) Vcp/p97-mediated unfolding as a principle in protein homeostasis and signaling. Mol. Cell 69, 182–194 CrossRef Medline
3. Ye, Y., Tang, W. K., Zhang, T., and Xia, D. (2017) A mighty "protein extractor" of the cell: structure and function of the p97/cdc48 ATPase. Front. Mol. Biol. 4, 39 CrossRef Medline
4. Meyer, H., Bug, M., and Bremer, S. (2012) Emerging functions of the vcp/p97 AAA-ATPase in the ubiquitin system. Nat. Cell Biol. 14, 117–123 CrossRef Medline
5. Chapman, E., Maksim, N., de la Cruz, F., and La Clair, J. J. (2015) Inhibitors of the AAA⁺ chaperone p97. Molecules 20, 3027–3049 CrossRef Medline
6. Deshaies, R. J. (2014) Proteotoxic crisis, the ubiquitin-proteasome system, and cancer therapy. BMC Biol. 12, 94 CrossRef Medline
7. DeLaBarre, B., and Brunger, A. T. (2003) Complete structure of p97/valosin-containing protein reveals communication between nucleotide domains. Nat. Struct. Mol. Biol. 10, 856–863 CrossRef Medline
8. Hänzelmann, P., and Schindelin, H. (2016) Structural basis of ATP hydrolysis and interubunit signaling in the AAA⁺ ATPase p97. Structure 24, 127–139 CrossRef Medline
9. Wang, Q., Song, C., and Li, C. C. (2013) Hexamerization of p97-vcp is promoted by ATP binding to the d1 domain and required for ATPase and biological activities. Biochem. Biophys. Res. Commun. 300, 253–260 CrossRef Medline
10. Wang, Q., Song, C., Yang, X., and Li, C. C. (2003) D1 ring is stable and nucleotide-independent, whereas d2 ring undergoes major conformational changes during the ATPase cycle of p97-vcp. J. Biol. Chem. 278, 32784–32793 CrossRef Medline
11. Wendler, P., Ciniaawy, S., Kock, M., and Kube, S. (2012) Structure and function of the AAA⁺ nucleotide binding pocket. Biochim. Biophys. Acta 1823, 1–14 CrossRef Medline
12. Song, C., Wang, Q., and Li, C. C. (2003) ATPase activity of p97-valosin-containing protein. J. Biol. Chem. 280, 40515–40523 CrossRef Medline
13. Chou, T. F., Bulfer, S. L., Weihl, C. C., Li, K., Lis, L. G., Walters, M. A., Milne, J. L., Huryn, D., Arkin, M., and Subramanian, S. (2016) 2.3 A resolution cryo-EM structure of human p97 and mechanism of allosteric inhibition. Science 351, 871–875 CrossRef Medline
14. DeLaBarre, B., and Brummer, A. T. (2003) Complete structure of p97/valosin-containing protein reveals communication between nucleotide domains. Nat. Struct. Mol. Biol. 10, 856–863 CrossRef Medline
15. Schuetz, A. K., and Kay, L. E. (2016) A dynamic molecular basis for malfunction in disease mutants of p97/vcp. Elife 5, e02143 CrossRef Medline
16. Tang, W. K., Li, D., Li, C. C., Esser, L., Dai, R., Guo, L., and Xia, D. (2010) A novel ATP-dependent conformation in p97 n-d1 fragment revealed by crystal structures of disease-related mutants. EMBO J. 29, 2217–2229 CrossRef Medline
17. Bodnar, N. O., Kim, K. H., Ji, Z., Wales, T. E., Svetlov, V., Nuclider, E., Engen, J. R., Walz, T., and Rapoport, T. A. (2018) Structure of the cdc48 ATPase with its ubiquitin-binding cofactor ubd1-npl4. Nat. Struct. Mol. Biol. 25, 616–622 CrossRef Medline
18. Blythe, E. E., Olson, K. C., Chau, V., and Deshaies, R. J. (2017) Ubiquitin- and ATP-dependent unfoldase activity of p97/vcp/npl4/ubd1 is enhanced by a mutation that causes multisystem proteinopathy. Proc. Natl. Acad. Sci. U.S.A. 114, 4380–4388 CrossRef Medline
19. Briggs, L. C., Baldwin, G. S., Miyata, N., Kondo, H., Zhang, X., and Freemont, P. S. (2008) Analysis of nucleotide binding to p97 reveals the properties of a tandem aaa hexameric ATPase. J. Biol. Chem. 283, 13745–13752 CrossRef Medline
20. Blythe, E. E., Olson, K. C., Chau, V., and Deshaies, R. J. (2017) Dynamic molecular basis for malfunction in disease mutants of p97/vcp. Elife 5, e02143 CrossRef Medline
21. Wang, Q., Tang, W. K., and Xia, D. (2003) D1 ring is stable and nucleotide-independent, whereas d2 ring undergoes major conformational changes during the ATPase cycle of p97-vcp. J. Biol. Chem. 278, 32784–32793 CrossRef Medline
22. Anderton, D. J., Le Moigne, R., Djakovic, S., Kumar, B., Rice, J., Wong, S., Anderson, D. J., Le Moigne, R., Djakovic, S., Kumar, B., Rice, J., Wong, S., and Freemont, P. S. (2012) The role of the n-domain in the ATPase activity of the mammalian AAA ATPase p97/vcp. J. Biol. Chem. 283, 30289–30299 CrossRef Medline
23. Li, X., Wang, J., Yao, B., Valle, E., Kiss von Soly, S., Madriaga, A., Soriano, F., and Weihl, C. C. (2008) Mutations in the human AAA(+) ATPase p97 as an approach to treat cancer through disruption of protein homeostasis. Cancer Cell 18, 5083–5096 CrossRef Medline
24. Mei, H., and Weihl, C. C. (2014) The vcp/p97 system at a glance: connecting cellular function to disease pathogenesis. J. Cell Sci. 127, 3877–3883 CrossRef Medline
25. Tang, W. K., and Xia, D. (2016) Mutations in the human AAA(+) chaperone p97 and related diseases. Front. Mol. Biol. 3, 79 CrossRef Medline
26. Watts, G. D., Wymers, J., Kovach, M. J., Mehta, S. G., Darvish, D., Pestrkon, A., Whyte, M. P., and Kimonis, V. E. (2004) Inclusion body myopathy associated with Paget disease of bone and frontotemporal dementia is caused by mutant valosin-containing protein. Nat. Genet. 36, 377–381 CrossRef Medline
27. Bulfer, S. L., Chou, T. F., and Arkin, M. R. (2016) p97 disease mutations modulate nucleotide-induced conformational change to alter protein-protein interactions. ACS Chem. Biol. 11, 2112–2116 CrossRef Medline
28. Niwa, H., Ewens, C. A., Tsang, C., Yeung, H. O., Zhang, X., and Freemont, P. S. (2012) The role of the n-domain in the ATPase activity of the mammalian AAA ATPase p97/vcp. J. Biol. Chem. 283, 30289–30299 CrossRef Medline
29. Manno, A., Noguchi, M., Fukushima, J., Motohashi, Y., and Kizukzu, A. (2010) Enhanced ATPase activities as a primary defect of mutant valosin-containing proteins that cause inclusion body myopathy associated with Paget disease of bone and frontotemporal dementia. Genes Cells 15, 911–922 Medline
30. Wendler, P., Ciniaawy, S., Kock, M., and Kube, S. (2012) Structure and function of the AAA(+) ATPase p97. J. Biol. Chem. 287, 8561–8570 CrossRef Medline
31. Weihl, C. C., Dalal, S., Pestrkon, A., and Hansson, P. I. (2006) Impaired protein aggregate handling and clearance underlie the pathogenesis of p97/vcp-associated disease. J. Biol. Chem. 283, 30289–30299 CrossRef Medline
32. Anderson, D. J., Le Moigne, R., Djakovic, S., Kumar, B., Rice, J., Wong, S., Wang, J., Yao, B., Valle, E., Kiss von Soly, S., Madriaga, A., Soriano, F., Menon, M. K., Wu, Z. Y., Kampmann, M., et al. (2015) Targeting the AAA ATPase p97 as an approach to treat cancer through disruption of protein homeostasis. Cancer Cell 28, 653–665 CrossRef Medline
33. Zhao, S., Wang, J., Yao, B., Wang, S., Djakovic, S., Kumar, B., Rice, J., Wong, S., Wang, J., Yao, B., Valle, E., Kiss von Soly, S., Madriaga, A., Soriano, F., Menon, M. K., Wu, Z. Y., Kampmann, M., et al. (2015) Targeting the AAA ATPase p97 as an approach to treat cancer through disruption of protein homeostasis. Cancer Cell 28, 653–665 CrossRef Medline
34. Magni, P., D’Alessio, R., Valasina, B., Avanzi, N., Rizzi, S., Asa, D., Gasparri, F., Cozzi, L., Cucchi, U., Orrenius, C., Polucci, P., Ballinari, D., and Biological activities. Biochem. Biophys. Res. Commun. 300, 253–260 CrossRef Medline
Perrera, C., Leone, A., Cervi, G., et al. (2013) Covalent and allosteric inhibitors of the ATPase vcp/p97 induce cancer cell death. Nat. Chem. Biol. 9, 548–556 CrossRef Medline

Bastola, P., Wang, F., Schaich, M. A., Gan, T., Freudenthal, B. D., Chou, T. F., and Chien, J. (2017) Specific mutations in the d1-d2 linker region of vcp/p97 enhance ATPase activity and confer resistance to vcp inhibitors. Cell Death Discov. 3, 17065 CrossRef Medline

Her, N. G., Toth, J. I., Ma, C. T., Wei, Y., Motamedchaboki, K., Sergienko, E., and Petroski, M. D. (2016) p97 composition changes caused by allosteric inhibition are suppressed by an on-target mechanism that increases the enzyme's ATPase activity. Cell Chem. Biol. 23, 517–528 CrossRef Medline

Ritz, D., Vuk, M., Kirchner, P., Bug, M., Schütz, S., Hayer, A., Bremer, S., Lusk, C., Baloh, R. H., Lee, H., Glatter, T., Gstaiger, M., Aebersold, R., Weihl, C. C., and Meyer, H. (2011) Endolysosomal sorting of ubiquitylated caveolin-1 is regulated by vcp and ubxd1 and impaired by vcp disease mutations. Nat Cell Biol. 13, 1116–1123 CrossRef Medline

Chou, T. F., and Deshaies, R. J. (2011) Quantitative cell-based protein degradation assays to identify and classify drugs that target the ubiquitin-proteasome system. J. Biol. Chem. 286, 16546–16554 CrossRef Medline

Dantuma, N. P., Lindsten, K., Glas, R., Jelline, M., and Masucci, M. G. (2000) Short-lived green fluorescent proteins for quantifying ubiquitin/proteasome-dependent proteolysis in living cells. Nat. Biotechnol. 18, 538–543 CrossRef Medline

Holohan, C., Van Schaeybroeck, S., Longley, D. B., and Johnston, P. G. (2013) Cancer drug resistance: an evolving paradigm. Nat. Rev. Cancer 13, 714–726 CrossRef Medline

Walker, J. E., Saraste, M., Runswick, M. J., and Gay, N. J. (1982) Distantly related sequences in the α- and β-subunits of atp synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. EMBO J. 1, 945–951 CrossRef Medline

Locke, M., Toth, J. I., and Petroski, M. D. (2014) Lys11- and Lys48-linked ubiquitin chains interact with p97 during endoplasmic reticulum-associated degradation. Biochem. J. 459, 205–216 CrossRef Medline