

Quantitative Cell-based Protein Degradation Assays to Identify and Classify Drugs That Target the Ubiquitin-Proteasome System^{*[5]}

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We have generated a set of dual-reporter human cell lines and devised a chase protocol to quantify proteasomal degradation of a ubiquitin fusion degradation (UFD) substrate, a ubiquitin ligase CRL2^{VHL} substrate, and a ubiquitin-independent substrate. Well characterized inhibitors that target different aspects of the ubiquitin-proteasome system can be distinguished by their distinctive patterns of substrate stabilization, enabling assignment of test compounds as inhibitors of the proteasome, ubiquitin chain formation or perception, CRL activity, or the UFD-p97 pathway. We confirmed that degradation of the UFD but not the CRL2^{VHL} or ubiquitin-independent substrates depends on p97 activity. We optimized our suite of assays to establish conditions suitable for high-throughput screening and then validated their performance by screening against 160 cell-permeable protein kinase inhibitors. This screen identified Syk inhibitor III as an irreversible p97/vasolin containing protein inhibitor (IC₅₀ = 1.7 μM) that acts through Cys-522 within the D2 ATPase domain. Our work establishes a high-throughput screening-compatible pipeline for identification and classification of small molecules, cDNAs, or siRNAs that target components of the ubiquitin-proteasome system.

The ubiquitin-proteasome system (UPS)³ comprises one of the most important mechanisms for post-translational regulation of protein function in eukaryotic cells. The UPS comprises hundreds of enzymes that promote covalent attachment of ubiquitin and UBL (ubiquitin-like) proteins to target proteins, as well as enzymes that reverse the modification. Conjugation of ubiquitin to target proteins is a multistep process (1–4). First, ubiquitin is primed for transfer by the ubiquitin-activating enzyme (E1), to which it becomes attached via a thioester

bond between the C terminus of ubiquitin and the active site cysteine of E1. Next, the activated ubiquitin is transferred from E1 to the active site cysteine of a ubiquitin-conjugating enzyme (E2). Finally, the E2~ubiquitin thioester binds a ubiquitin ligase (E3), and the ubiquitin is transferred to the side chain amino group of a lysine residue on a substrate protein that is simultaneously bound to the same E3. Either this reaction can either terminate, or additional ubiquitin transfers can occur to ubiquitin itself, resulting in formation of a chain of ubiquitins attached to the substrate. Attachment of ubiquitin or UBL proteins to a target can yield diverse outcomes, including a change in the activity, localization, binding partners, or stability of the target. The most intensively studied consequence of ubiquitination is protein degradation. Polymerization of a chain linked together via Lys-48 of ubiquitin typically results in rapid degradation of the modified target by the 26 S proteasome. The proteasome binds proteins bearing Lys-48-linked ubiquitin chains and degrades the modified protein while recycling the ubiquitin for future use. The proteasome also degrades a handful of proteins, including ornithine decarboxylase (ODC), in a ubiquitin-independent manner (5). Given the importance of the UPS to regulatory biology, there has been considerable interest in developing small molecule inhibitors as potential therapies for a range of human diseases. The UPS has been validated as an important target in cancer by clinical use of the proteasome inhibitor bortezomib (Velcade) for the treatment of multiple myeloma and mantle cell lymphoma (6, 7). The success of bortezomib has inspired interest in developing other UPS-directed drugs (8) that have greater efficacy but fewer side effects.

The AAA (ATPase associated with diverse cellular activities) ATPase p97 is conserved across all eukaryotes and is essential for life in budding yeast (9) and mice (10). p97 is overexpressed in several cancers, supporting the idea that it could be a target of general importance in oncology (11, 12). Loss-of-function studies in model organisms indicate that p97 plays a critical role in a broad array of cellular processes including Golgi membrane reassembly (13), membrane transport (14, 15), degradation of misfolded membrane and secretory proteins by the UPS (16, 17), regulation of myofibril assembly (18), cell division (19), and formation of protein aggregates (20–22). This broad range of functions is thought to derive from the ability of p97 to unfold proteins or disassemble protein complexes. Several factors make p97 an intriguing target for the development of drugs to treat cancer. First, elevated expression levels of p97 have been associated with poor prognosis of cancer (23, 24). Second, p97 is an ATP hydrolase, and thus in theory, it should be druggable.

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[5] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental "Methods," Figs. 1–6, Tables 1–3, and additional references.

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³ The abbreviations used are: UPS, ubiquitin-proteasome system; ODC, ornithine decarboxylase; ERAD, endoplasmic reticulum-associated degradation; CHX, cycloheximide; Luc, luciferase; DMSO, dimethyl sulfoxide; UFD, ubiquitin fusion degradation; Eer1, Eeyarestatin I; HTS, high-throughput screening; TCR, T-cell receptor.

Third, p97 is essential, and thus, p97 inhibitors should have antiproliferative activity. In addition, p97 is essential for endoplasmic reticulum-associated degradation (ERAD) (15, 25, 26). Blockade of ERAD is thought to be a key mechanism underlying the anticancer effects of bortezomib (27). Given that p97 is implicated in ERAD but otherwise has a more restricted role in the UPS compared with the proteasome, it is possible that drugs that target p97 might retain much of the efficacy of bortezomib but with less toxicity.

EXPERIMENTAL PROCEDURES

Materials—The antibodies used in this study were anti-GFP (BD Biosciences), anti-luciferase (Promega), and anti-p97 (Research Diagnostics). MG132 was purchased from BIOMOL. Cycloheximide (CHX) and 3,4-methylenedioxy- β -nitrostyrene (compound 7) was purchased from EMD Bioscience. 3,4-Methylenedioxy-cinnamic acid (compound 18) was purchased from Sigma. Thalidomide was purchased from AK Scientific. Cells were grown on a 96-well CELLSTAR black μ Clear bottom plate (ISC Bioexpress) for live cell imaging on an ImageXpress Micro automated microscope (Molecular Devices). Luciferase intensity was determined on an Analyst AD plate reader (Molecular Devices). The plasmids, primer sequences, cell lines, and siRNA sequences used in this study are listed in [supplemental Table 1](#). RDB2004 was subjected to site-directed mutagenesis using primers E305Q-F and E305Q-R to create QQ-p97 (RDB2405). RDB2120 was subjected to site-directed mutagenesis using primers C522A-F and C522A-R to create C522A-p97 (RDB2402).

Generation of Dual-reporter Stable Cell Lines—Lentiviruses were packaged by cotransfection with helper plasmids in 293T cells (DTC 20) (28) using FuGENE HD (Roche Applied Science). Ub^{G76V}-GFP-expressing HeLa cells (DTC 9) were infected with one of three lentiviruses expressing wild-type luciferase (Luc; RDB2392), ODD-Luc (RDB2391), or Luc-ODC (RDB2390) (29) and were grown in DMEM containing puromycin (2.5 μ g/ml) to establish stably transduced cell lines (DTC 23–25).

Reporter Accumulation Assay—Cells were seeded on 96-well plates (5000 cells/well) and grown for 16 h. Cells were treated with modified DMEM (without phenol red, folic acid, riboflavin, and vitamin B₁₂) containing dimethyl sulfoxide (DMSO), MG132, or a test compound for 2–3 h. Two GFP images per well with 100-ms exposure time were acquired by the automated microscope, and the average GFP intensity per area of a cell was determined using the cell scoring function of MetaXpress software. The mean GFP intensity of \sim 500 cells was calculated using Excel. Cells without the GFP reporter were used to do background correction. The effect of MG132 was calculated by taking the normalized GFP intensity for cells treated with MG132 and dividing by the same value obtained from DMSO-treated cells. Luciferase assays were carried out in 96-well white solid bottom plates. D-Luciferin (50 μ l of 1 mg/ml in PBS) was added into each well containing 50 μ l of medium and incubated at room temperature with shaking at 500 rpm for 5 min. Luminescence intensity was determined with 0.1-ms integration time.

Reporter Degradation Assay—Cells were seeded on 96-well plates (5000 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 μ M) for 1 h and washed twice with prewarmed PBS (100 μ l). Modified DMEM containing FBS (2.5%), CHX (30 μ g/ml), and DMSO or a test compound (0–30 μ M) was added to each well. Plates were imaged on the ImageXpress Micro microscope at different time points. For the ODD-Luc degradation assay, four identical 96-well white solid bottom plates were prepared. At each time point, one plate was taken out of the incubator, and luciferase activity was determined as described for the reporter accumulation assay. Detailed methods for data analysis are described under [supplemental “Methods.”](#)

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 according to the manufacturers' protocols. Materials and additional methods are described under [supplemental “Methods.”](#)

RESULTS AND DISCUSSION

Quantitative CHX Chase Assay in Live Cells to Monitor UPS Function—Three dual-reporter stable HeLa cell lines were generated to monitor UPS function in mammalian cells. One expressed the well characterized ubiquitin fusion degradation (UFD) reporter Ub^{G76V}-GFP (30) and a recently described construct that features the oxygen-dependent degradation domain of HIF1 α fused to luciferase (ODD-Luc) (29). The second expressed Ub^{G76V}-GFP and luciferase fused to the ubiquitin-independent degradation domain of ornithine decarboxylase (Luc-ODC) (29). Schematic diagrams of these and other reporters used in this work are shown in [supplemental Fig. 1](#). The third line expressed Ub^{G76V}-GFP and unfused luciferase. As expected based on prior work, the Ub^{G76V}-GFP and ODD-Luc reporters accumulated by \sim 20-fold in cells treated with 7.5 μ M MG132 for 2 h (Fig. 1A). However, the Luc-ODC reporter exhibited only a modest 1.6-fold accumulation upon exposure to 30 μ M MG132 for 2.5 h (Fig. 1B), which is consistent with both the longer half-life of Luc-ODC (see below) and a previous report (29). The unfused luciferase was used to weed out compounds that affected luciferase activity.

Increases in the steady-state level of a protein depend on rates of both protein synthesis and degradation. Therefore, genetic manipulations or drugs that inhibit degradation might give a misleading result if they also influence gene expression or protein synthesis. For this reason, pulse-chase experiments are widely acknowledged to be the gold standard for monitoring protein degradation. However, published uses of the optically active reporters described above have relied almost exclusively on monitoring reporter accumulation as opposed to reporter degradation (29–33). A potential problem with this approach was noted by Alvarez-Castelao *et al.* (34), who demonstrated that accumulation of three UPS reporters in response to proteasome inhibitors is driven in large measure by up-regulation of the constructs' cytomegalovirus promoter. To diminish the potential for “off-target” effects, we developed an assay to evaluate specifically the effect of genetic perturbations or drugs on

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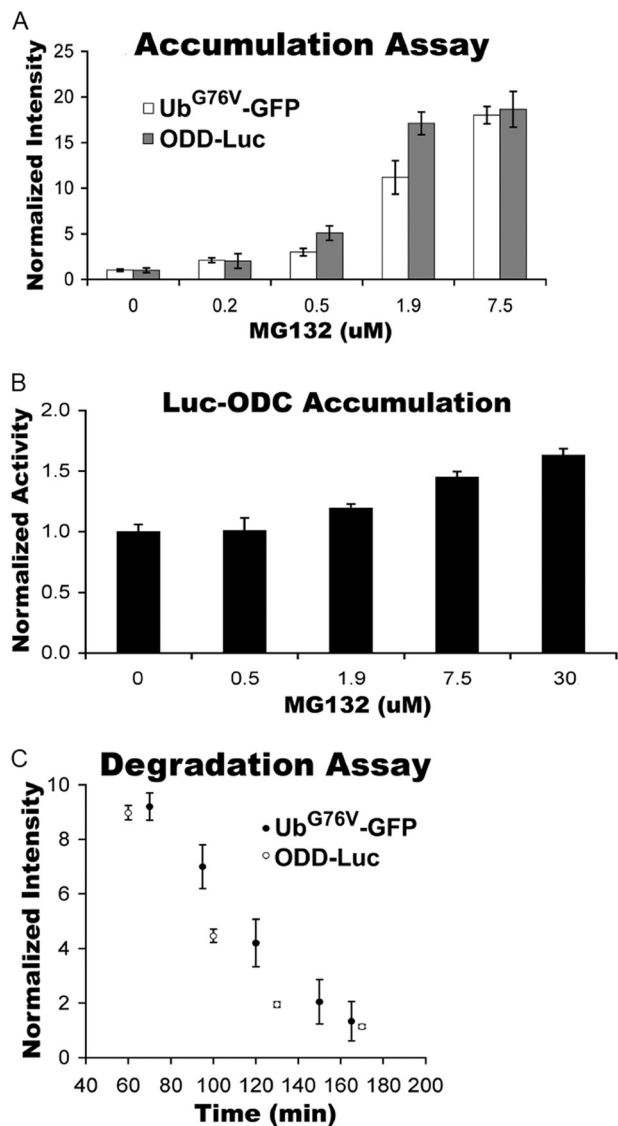


FIGURE 1. Development of dual-reporter cell lines and a CHX chase assay to monitor UPS function. *A*, HeLa cells stably expressing Ub^{G76V}-GFP and ODD-Luc were treated with the indicated amounts of MG132 for 2 h. Signal intensities for GFP and luciferase accumulation were normalized to values obtained with DMSO-treated cells. *B*, same as *A*, except that cells expressing Ub^{G76V}-GFP and Luc-ODC were used. *C*, the degradation rates of Ub^{G76V}-GFP and ODD-Luc were determined by first accumulating the proteins in MG132 (4 μM for 1 h) and then washing out the drug and monitoring the rate of signal decay in a CHX chase.

protein degradation. A commonly used assay to monitor protein degradation in eukaryotic cells is the “cycloheximide chase,” wherein CHX is added to cells, and the decay in the steady-state level of a target protein is monitored by immunoblotting. Unfortunately, it was initially not possible to use this simple approach to monitor Ub^{G76V}-GFP degradation due to the nearly undetectable steady-state levels of this reporter in unperturbed cells. Accordingly, we first increased the initial level of Ub^{G76V}-GFP by reversibly inhibiting the proteasome with MG132 (35). Because of the extremely rapid turnover of Ub^{G76V}-GFP, even a very brief incubation (1–2 h) with MG132 was sufficient to yield a readily detectable signal. Importantly, most established cell lines can withstand treatment with proteasome inhibitors for many hours (36), and thus, a 1-h treat-

ment is anticipated to have a minimum effect on physiology and does not elicit detectable induction of the apoptotic pathway (37). We then removed the MG132 and initiated a classical CHX chase, which allowed us to monitor the half-life of reporter degradation in the absence of confounding synthesis. GFP intensity was monitored at the time of removal of MG132 (time = 0 min) and every 20–25 min thereafter (Fig. 1*C*, closed circles). The degradation rate constant (k) and half-life ($0.693/k$) were obtained from the slope of plotting $\ln(\text{normalized GFP intensity})$ versus time (supplemental Fig. 2), starting 60 min after initiation of the chase. ODD-Luc degradation assayed under the same conditions yielded a similar curve (Fig. 1*C*, open circles). Note that it was not necessary to pre-accumulate ODD-Luc or Luc-ODC with MG132 due to much more sensitive detection of luciferase activity compared with fluorescence. Thus, in subsequent experiments (Fig. 2, *B–E*), pre-accumulation with MG132 was not employed. A representative example of how we determined the half-maximal inhibitory concentration (IC_{50}) of MG132 in the Ub^{G76V}-GFP degradation assay is shown in supplemental Fig. 2.

Validation of Dual-reporter Cell Lines with Published Chemical Inhibitors of the UPS—To benchmark our dual-reporter HeLa cells, we evaluated their responses to known chemical inhibitors of the UPS, including a reversible proteasome inhibitor (MG132 (35)), a covalent proteasome inhibitor (YU101) (38), a covalent ubiquitin E1 inhibitor (PYR-41) (39), a CRL^{CRBN} ubiquitin ligase complex inhibitor (thalidomide) (40), an SCF^{Met30} inhibitor (SMER3) (41), a p53-HDM2 inhibitor (JNJ26854165) (42), and a Nedd8 E1 inhibitor (MLN4924) (43) (Table 1). MG132 inhibited degradation of the Ub^{G76V}-GFP and ODD-Luc reporters with similar IC_{50} values, whereas the IC_{50} values obtained with the Luc-ODC reporter were ~3-fold higher. YU101 behaved very similarly to MG132. PYR-41 inhibited both Ub^{G76V}-GFP and ODD-Luc degradation but had no effect on Luc-ODC degradation, which is consistent with ODC being a ubiquitin-independent proteasome substrate (5). Meanwhile, MLN4924 elicited strong stabilization of ODD-Luc but had no effect on the other reporters (Table 1 and supplemental Fig. 3*A*). This result is consistent with ODD-Luc being a substrate for the ubiquitin ligase CRL2^{VHL}, the activity of which is dependent on Nedd8 conjugation (44). The results with MG132, PYR-41, and MLN4924 indicated that our reporters behaved exactly as predicted. The different reporter assays allowed us to generate a rubric that can be used to narrow the field of targets for any chemical inhibitor of the UPS (Table 2). Interestingly, our findings expand the range of biological activity of SMER3 and JNJ26854165 beyond the targets that were originally identified for these compounds. The ability to define in greater depth the activity of these compounds toward the UPS targets underscores the utility of our suite of assays.

Using our rubric as a guide, we sought to evaluate a recently described inhibitor of p97 complexes, Eeyarastatin I (EerI) (45). EerI blocked degradation of Ub^{G76V}-GFP with an IC_{50} of 3.7 μM (supplemental Fig. 4) (37), which is consistent with the published report that this reporter accumulates in cells depleted of p97 (31). Unfortunately, we could not assay EerI in high-throughput format on the ODD and ODC reporters because it

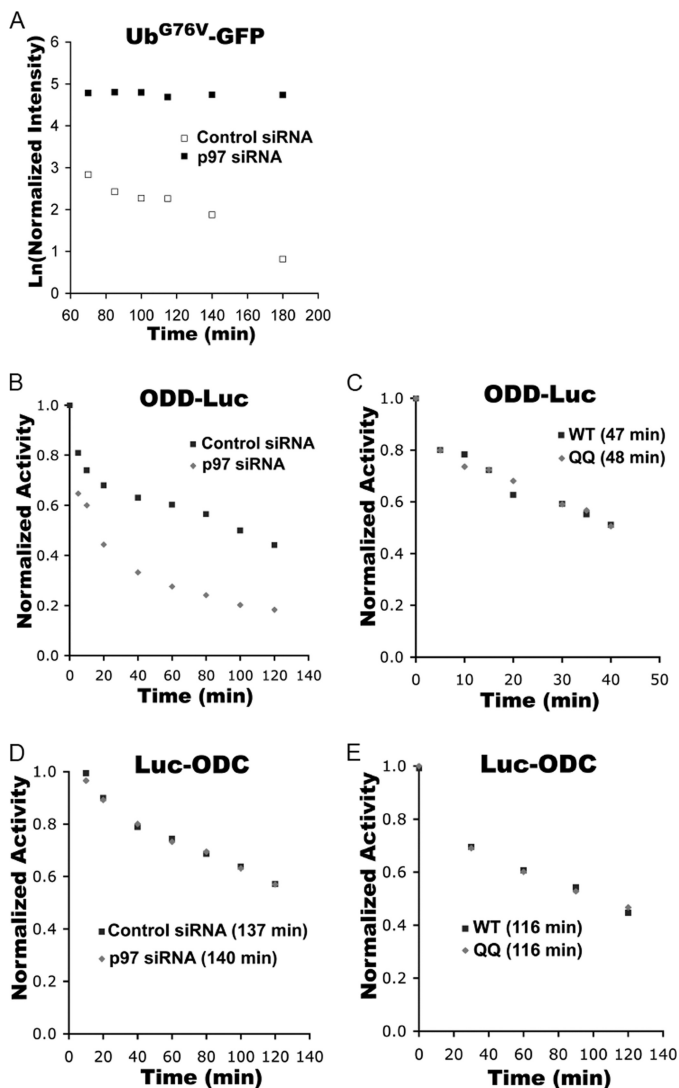


FIGURE 2. Degradation of luciferase reporters is independent of p97. To evaluate the effect of p97 on three reporters, cells were transfected with control or p97 siRNA for 48 h. For overexpression of the ATPase-inactive mutant of p97, cells were transiently transfected with QQ-p97 or WT p97 expression plasmids and analyzed after 24 h. A, Ub^{G76V}-GFP degradation was measured by CHX chase (see “Experimental Procedures”) in cells treated with siRNAs directed against either luciferase (control) or p97. Successful depletion of p97 was confirmed by immunoblotting (see [supplemental Fig. 4B](#)). B–E, degradation of basal ODD-Luc (B and C) or Luc-ODC (D and E) was measured by CHX chase assay (without preincubation in MG132) using dual-reporter cells that were pretreated as follows. B and D, cells were transfected with siRNA directed against p97 or non-silencing negative control. C and E, cells were transfected with plasmids encoding either WT p97 or dominant-negative p97 (QQ-p97).

interfered with luciferase activity. Therefore, we assayed the effect of EerI on ODD-Luc degradation by Western blotting ([supplemental Fig. 3B](#)). EerI did not block ODD-Luc degradation even when assayed at a concentration 5-fold higher than its IC₅₀ for inhibiting Ub^{G76V}-GFP degradation. These data are consistent with EerI being a selective inhibitor of p97 or its cofactors (see below for further justification of this conclusion).

Dual-reporter Cell Lines Can Be Used to Distinguish p97-dependent and p97-independent Degradation—Our observations with EerI suggested that we could identify molecules that selectively block p97 activity but not other general components of

the UPS (e.g. proteasome and E1 enzyme) by seeking compounds that stabilize Ub^{G76V}-GFP but not ODD-Luc. To validate this hypothesis, we determined the degree to which the reporters were stabilized by knocking down endogenous p97 with siRNA or overexpressing the ATPase-deficient mutant of p97 (QQ-p97). Definitive confirmation of the p97 dependence of Ub^{G76V}-GFP degradation was obtained from a CHX chase experiment. Depleting p97 or expressing the QQ-p97 mutant increased the half-life of Ub^{G76V}-GFP by 14–28-fold (Fig. 2A and [supplemental Table 2](#)). By contrast, the ODD-Luc and Luc-ODC reporters behaved quite differently (Fig. 2, B–E). ODD-Luc and Luc-ODC were not stabilized by either siRNA-mediated depletion of p97 or expression of QQ-p97 (see [supplemental Table 1](#) for a summary of half-lives). The failure of p97 siRNA to stabilize ODD-Luc was confirmed by Western blotting ([supplemental Fig. 5, lanes 1–4](#)). It is not presently known why some proteins (e.g. Ub^{G76V}-GFP) depend on p97 for their degradation, whereas others (such as ODD-Luc) do not. It is thought that, for some substrates, p97 dependence may be related to the exposure of unstructured regions (31). Regardless of the underlying reason, Ub^{G76V}-GFP and ODD-Luc are convenient tools for monitoring p97-dependent and p97-independent degradation within the UPS.

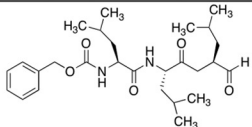
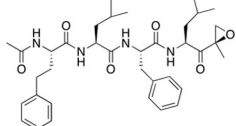
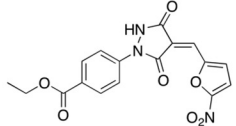
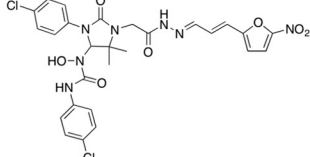
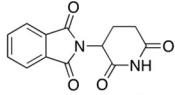
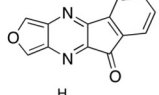
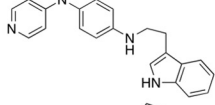
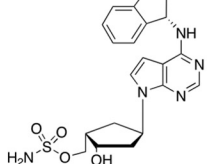
Screening of 160 Cell-permeable Protein Kinase Inhibitors with Dual Reporters—To demonstrate the utility of our dual-reporter cell lines for high-throughput screening (HTS) assay, we first optimized multiwell plate-based assays to monitor accumulation and degradation of both Ub^{G76V}-GFP and ODD-Luc. Three quality control parameters were calculated: (a) the signal/base line ratio, (b) the coefficient of variation (S.D./mean) × 100, and (c) the Z'-factor $(1 - ((3 \times (\sigma_P + \sigma_N)) / (\mu_P - \mu_N)))$, where σ is the standard deviation and μ is the mean for positive (P) and negative (N) controls (46). MG132 (2 μ M) was used as a positive control, and DMSO was used as a negative control. In the 96-well plate format, all assays exhibited a Z'-factor >0.5 (Table 3), which qualifies an assay for HTS according to metrics established by the National Institutes of Health for screens conducted within the Molecular Libraries Probe Production Centers Network (grants.nih.gov/grants/guide/pa-files/PAR-10-182.html#SectionI). The optimum duration for both assays was 3 h after the addition of compounds, and both assays allowed repetitive imaging of the same plate at different time points to determine the kinetics of signal change. A representative image from a 3-h Ub^{G76V}-GFP degradation assay is shown in [supplemental Fig. 6](#).

We next screened a collection of 160 cell-permeable protein kinase inhibitors in 96-well plates using the degradation assay (Fig. 3A and [supplemental Table 3A](#)). The rationale underlying this screen is that protein kinase inhibitors typically target the ATP-binding site, and we reasoned that some known kinase inhibitors might cross-inhibit one of the ATPase active sites of p97. We first evaluated the inhibitory effect of library compounds on Ub^{G76V}-GFP degradation at 12 μ M. Thirty-four compounds that exhibited >50% inhibition were subjected to 7-point titrations (4-fold serial dilutions starting at 30 μ M), which yielded 29 compounds with IC₅₀ values <20 μ M. These compounds were further evaluated by Western blotting with anti-GFP antibody to eliminate false positives due to com-

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TABLE 1

Published chemical inhibitors of the UPS

Compound ID	Structure	Reported Target	Assay IC ₅₀ (μM)*		
			Ub ^{G76V} -GFP Degradation	ODD-Luc Degradation	Luc-ODC Degradation
MG132		Proteasome	0.27 ± 0.03	0.30 ± 0.04	1.2 ± 0.2
YU101		Proteasome	0.35 ± 0.03	1.5 ± 0.4	2.5 ± 0.6
PYR-41		Ubiquitin-activating enzyme E1	7.0 ± 2.0	15 ± 5	> 30
Eeyarestatin I (EerI)		p97 and/or ataxin 3	3.7 ± 0.4 **	NM***	NM
(+/-)-Thalidomide		CRL4 ^{CRBN}	>50	>50	>50
SMER3		SCF ^{Met30}	1.0 ± 0.2	1.0 ± 0.4	1.0 ± 0.3
JNJ26854165		p53-HDM2	14±3	NM	NM
MLN4924		Nedd8 activating enzyme (NAE)	>30	0.05±0.01	>30

*, Measurements were carried out in triplicate, and variance is expressed as the standard deviation. **, data were adapted from Ref. 37. Measurements were carried out in triplicate, and variance is expressed as S.D. ***, NM, not measured due to interference with luciferase activity.

TABLE 2

Classification of potential targets for chemical inhibitors on the basis of their pattern of reporter stabilization

Reporter stabilization ^a			Potential target	Compound evaluated ^b
Ub ^{G76V} -GFP	ODD-Luc	Luc-ODC		
+	+	+	Proteasome or other general UPS factor that is not ubiquitin-specific	MG132, YU101, SMER3
+	+	-	E1 enzyme or other Ub-specific factor	PYR-41
-	+	-	CUL2 ^{VHL} or general cullin-RING ligase component	MLN4924
-	-	-	Not specified by these assays	Thalidomide
+	-	-	p97 or the UFD pathway	EerI ^c

^a +, stabilization of the reporter; -, little or no effect.

^b EerI and JNJ26854165 were difficult to categorize using this suite of assays due to their interference with luciferase activity.

^c Although EerI could not be evaluated using a luciferase readout, immunoblotting confirmed that it behaves as an inhibitor of the p97-UFD pathway (*i.e.* no accumulation or stabilization of ODD-Luc and Luc-ODC reporters).

pound autofluorescence (14 compounds). The remaining 15 active compounds were assayed for their ability to inhibit degradation of ODD-Luc and Luc-ODC, as well as their effects on

p97 ATPase and 26 S proteasome activities *in vitro*. The assay results for these compounds plus two autofluorescent compounds that blocked Ub^{G76V}-GFP degradation as determined

TABLE 3
Summary of HTS quality control parameters for 96-well plates

Parameter	Accumulation		Degradation	
	Ub ^{G76V} -GFP reporter	ODD-Luc reporter	Ub ^{G76V} -GFP reporter	ODD-Luc reporter
S/B ^a	3.4	11	2.6	5.8
CV ^b	3.3% (P), 1.9% (N)	7.3% (P), 4.5% (N)	7.4% (P), 7.3% (N)	8.2% (P), 7.6% (N)
Z'-factor ^c	0.67	0.7	0.5	0.57

^a S/B, signal/base line ratio.

^b CV, coefficient of variation ((S.D./mean) × 100).

^c Z'-factor = 1 - ((3 × (σ_P + σ_N))/(μ_P - μ_N)), where σ is S.D. and μ is the mean for positive (P) and negative (N) controls. The positive control was MG132 (2 μM), and the negative control was DMSO (0.8%).

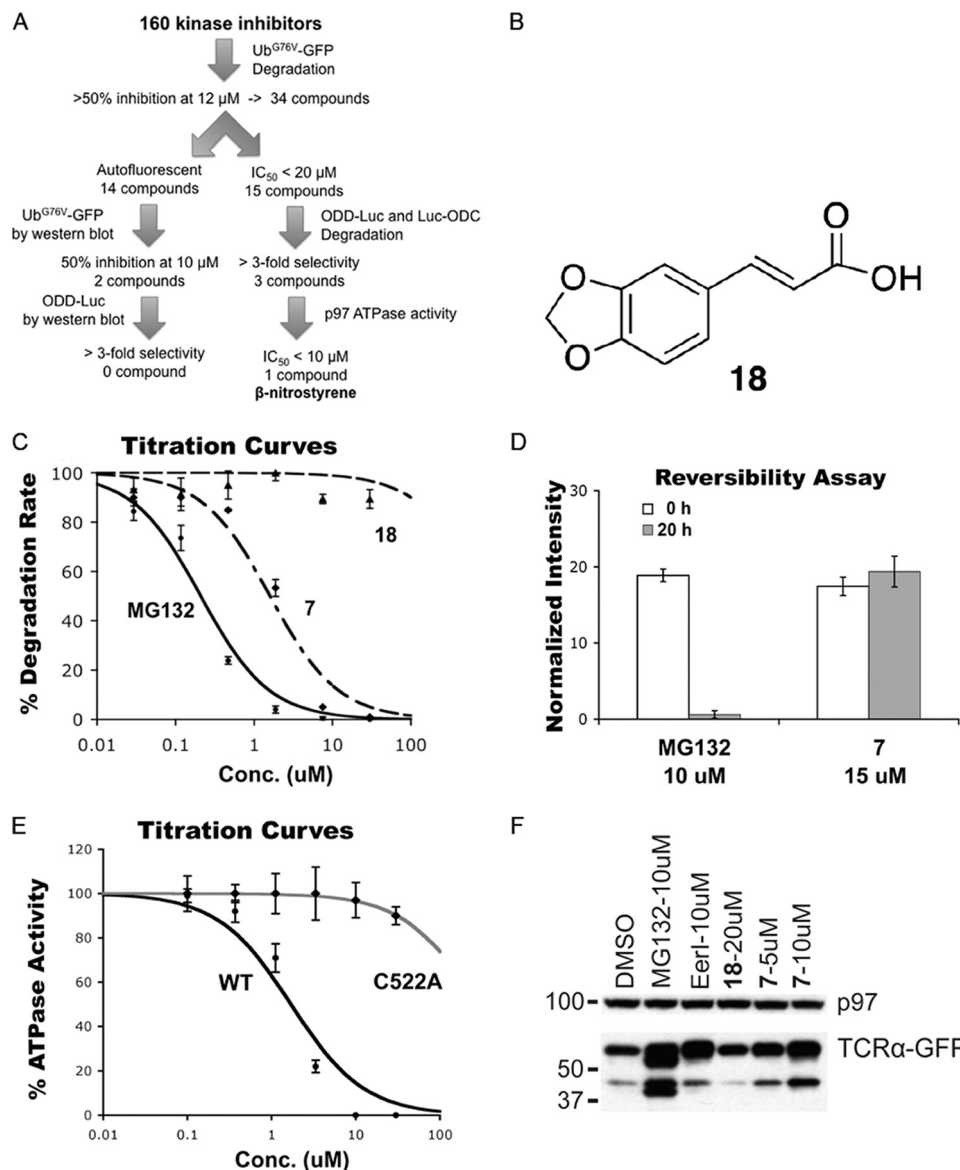


FIGURE 3. Identification of 3,4-methylenedioxy-β-nitrostyrene (compound 7) as an inhibitor of Ub^{G76V}-GFP and TCRα-GFP degradation. *A*, summary of the multistep screen of 160 protein kinase inhibitors. *B*, structure of compound **18**. *C*, titration curves of MG132 and compounds **7** and **18** for inhibiting Ub^{G76V}-GFP degradation. *D*, the reversibility of compound inhibition was determined by first accumulating Ub^{G76V}-GFP in the presence of MG132 (4 μM for 1 h), washing out MG132, and exposing cells to CHX plus MG132 (10 μM) or compound **7** (15 μM) for 2 h and then washing out the test compound and monitoring decay of the GFP signal in CHX for 20 h. *E*, titration curves for inhibition of the *in vitro* ATPase activity of WT p97 (black curve) or C522A-p97 (gray curve) by compound **7**. *F*, HEK293 cells stably expressing the ERAD reporter TCRα-GFP were used to determine the effect of compound **7** on the ERAD pathway. Cells were treated with MG132, washed, and then incubated in the presence of CHX plus test compound for 2 h prior to harvest. Samples were immunoblotted with anti-GFP antibody to detect TCRα-GFP. p97 served as a loading control.

by Western blotting are listed in Table 4 and supplemental Table 3B. Several noteworthy findings emerged from this analysis. First, at least 8% (13/160) of the compounds in the com-

mercial library we screened inhibited degradation of at least one of our reporters with an IC₅₀ of ≤10 μM. By contrast to the results obtained in the degradation assays, only one compound

TABLE 4

Summary of 17 hits from the kinase inhibitor screen

Compound	PubChem CID	Description	IC ₅₀ (μM) ^a				
			Ub ^{G76V} -GFP	ODD-Luc	Luc-ODC	p97 ATPase ^b	Proteasome ^c
1	5719375	Akt inhibitor IV	10 ± 2	5.0 ± 0.5	12 ± 5	12 ± 5	1.5 ± 0.3
2	10196499	Akt inhibitor VIII	3.7 ± 0.9	3.6 ± 0.5	2.9 ± 0.4	43 ± 4	3.7 ± 0.9
3	16760284	Akt inhibitor X	16 ± 9	14 ± 11	11 ± 5	>50	40 ± 8
4	5113385	PDK1/Akt/Flt	0.52 ± 0.12	3.4 ± 0.9	3 ± 1	>50	>30
5	72311	Chelerythrine chloride	2.2 ± 1	3.4 ± 0.2	1.9 ± 0.7	24 ± 11	48 ± 8
6	16760502	Herbimycin A, <i>Streptomyces</i> sp.	2.8 ± 1.6	5 ± 2	>30	>50	>30
7	672296	Syk inhibitor III	1.6 ± 0.4	5.9 ± 1.5	>30	1.7 ± 0.5	162 ± 41
8	451705	Staurosporine, <i>Streptomyces</i> sp.	12 ± 2.0	19 ± 4	16 ± 4	>50	8.0 ± 1.5
9	5353431	BAY 11-7082	3.0 ± 0.8	0.6 ± 0.08	0.5 ± 0.1	12 ± 1	>30
10	2794188	Cdk1 inhibitor	7.0 ± 1.0	15 ± 2	7.0 ± 2.0	17 ± 3	28 ± 5
11	481747	Cdk4 inhibitor III	2.8 ± 0.9	8.0 ± 2.0	7.2 ± 2.3	13 ± 0.5	>30
12	73292	Fascaplysin	0.9 ± 0.1	6.9 ± 1.9	>30	>50	73 ± 14
13	5287844	GSK-3 inhibitor IX	12 ± 4	8.2 ± 2	1.0 ± 0.1	>50	32 ± 8
14	11665831	JNK inhibitor, negative control	3.8 ± 0.8	0.15 ± 0.01	0.11 ± 0.01	>50	>30
15	490561	K-252a, <i>Nocardioopsis</i> sp.	10 ± 4	7.2 ± 1.4	2.5 ± 0.7	26 ± 2	9.2 ± 3.1
16	6711154	PKCβII/EGFR ^d inhibitor	~10	~10	ND ^d	>50	>30
17	9549300	Tpl2 kinase inhibitor	~10	~10	ND	>50	>30

^a Measurements were carried out in triplicate, and variance is expressed as S.D.

^b The p97 ATPase assay is described under supplemental "Methods."

^c The human 26 S proteasome assay is described under supplemental "Methods."

^d EGFR, EGF receptor; ND, not determined.

(7, Syk inhibitor III) inhibited p97 ATPase with an IC₅₀ of ≤10 μM. Second, although most compounds exhibited similar IC₅₀ values toward the three reporters, there were some interesting differences. For example, herbimycin A (compound 6), Syk inhibitor III (compound 7), and fascaplysin (compound 12) exhibited very little activity in the Luc-ODC assay, implying that they target some aspect of ubiquitin-dependent degradation. Meanwhile, two compounds (JNK inhibitor negative control (compound 14) and BAY 11-7082 (compound 9)) were considerably more potent in stabilizing the luciferase reporters, implying that, in some unknown manner, they may stabilize luciferase against proteasome-dependent turnover. Third, compounds 1–4, which target Akt, stabilized all three reporters but appeared to act by different mechanisms. Whereas compounds 1 and 2 inhibited ATP-dependent 26 S proteasome chymotrypsin-like activity with submicromolar IC₅₀ values, compounds 3 and 4 did not. This suggests the interesting possibility that compounds 1 and 2 may be inhibitors of one or more of the six proteasomal ATPases. Taken together, our observations on a small collection of kinase inhibitors highlight the potential risk in interpreting results obtained with small molecules before thoroughly evaluating their potential off-target effects.

Identification of an Irreversible p97 Inhibitor—Compound 7 (3,4-methylenedioxy-β-nitrostyrene) (47, 48) was repurchased for a more detailed analysis due to its low IC₅₀ values in both the Ub^{G76V}-GFP degradation and *in vitro* ATPase assays and its 3.5-fold lower IC₅₀ for p97-dependent versus p97-independent reporters. Replacing the nitro group with a carboxylic acid (compound 18) (Fig. 3B) greatly diminished activity (Fig. 3C), suggesting that the nitro group was critical for the observed inhibition. This implied that the primary mechanism of action was covalent. Moreover, Ub^{G76V}-GFP was degraded in cells treated with MG132 for 2 h and then transferred to fresh medium containing CHX for 20 h but remained stable in cells pretreated with compound 7 prior to CHX chase (Fig. 3D), providing further support for the idea that the mechanism of inhibition was irreversible. Electrophilic agents that react with

a nonessential cysteine (Cys-522) in the D2 domain active site can irreversibly inhibit p97 (49). Therefore, we compared the activity of compound 7 toward wild-type p97 and C522A-p97. The poor activity of compound 7 toward the C522A mutant suggested that electrophilic attack of Cys-522 is indeed critical for the potency of compound 7 (Fig. 3E). p97 is perhaps best known for its role in promoting ERAD (14). If compound 7 inhibits p97 in cells, it should block ERAD. To test this prediction, we evaluated the impact of compound 7 on the ERAD reporter TCRα-GFP (α-chain of the T-cell receptor fused to GFP). TCRα-GFP overexpressed in non-T-cells inserts into the endoplasmic reticulum but behaves as an unfolded protein and is degraded by the proteasome in a p97-dependent manner (32). Consistent with its p97 inhibitory activity, compound 7 potentially blocked degradation of TCRα-GFP at 5 or 10 μM (Fig. 3F).

Compound 7 Exhibits Distinct Behavior in Degradation Versus Accumulation Assay—Our initial rationale for developing a high-throughput degradation assay was to identify small molecules or genetic constructs that block protein degradation independent of any effects they may have on mRNA transcription or translation. To evaluate the potential for effects on reporter accumulation to obscure effects on reporter degradation, we rescreened the collection of 160 kinase inhibitors to identify those compounds that promoted accumulation of Ub^{G76V}-GFP. Interestingly, of those compounds shown in Table 4, compounds 3, 7, and 10–12 did not score as positives in the accumulation assay. To evaluate this discrepancy in more depth, we measured the concentration-dependent accumulation of Ub^{G76V}-GFP in response to compound 7 or its inactive analog, compound 18 (Fig. 4A). When tested at 7.5 or 30 μM, compound 7 elicited <2-fold accumulation of Ub^{G76V}-GFP. By contrast, the same compound caused 2-fold stabilization at 1.7 μM and almost complete stabilization at 7.5 μM (Fig. 3C). Thus, the degradation assay was much more responsive to compound 7, suggesting that this compound may inhibit some step in expression of the Ub^{G76V}-GFP reporter. To test this hypothesis, we assayed accumulation of

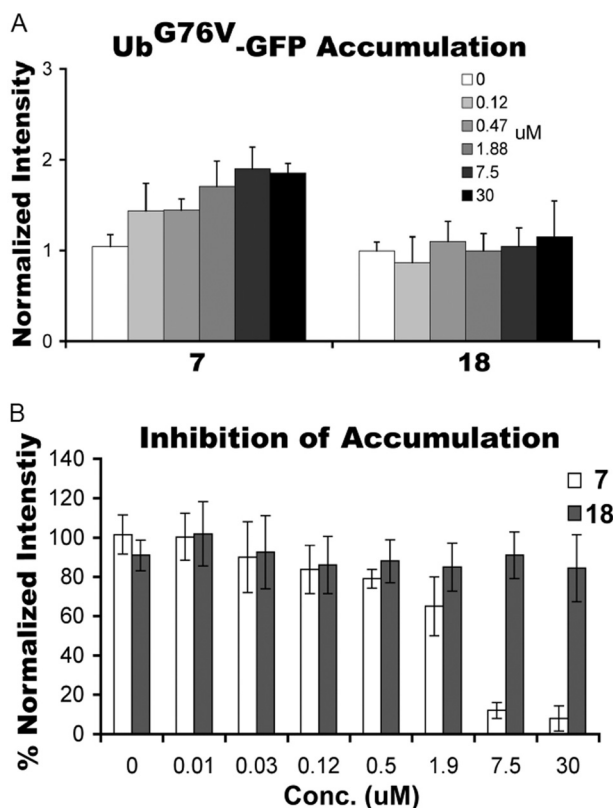


FIGURE 4. Compound 7 inhibits MG132-dependent Ub^{G76V}-GFP accumulation. *A*, concentration-dependent accumulation of Ub^{G76V}-GFP by compounds **7** and **18** after 3 h of treatment. GFP intensity was normalized to values obtained with DMSO-treated cells. *B*, HeLa cells expressing Ub^{G76V}-GFP were co-incubated with MG132 (4 μM) and increasing amounts of compound **7** or **8** for 3 h. GFP intensity was normalized to values obtained with MG132-treated cells.

Ub^{G76V}-GFP in the presence of 4 μM MG132 and compound **7** or **18** (Fig. 4*B*). Compound **7** inhibited MG132-induced accumulation of the reporter with an IC₅₀ of 2.1 ± 0.5 μM, which explains why we observed only a small effect in the accumulation assay (Fig. 4*A*).

In conclusion, we have described a set of dual-reporter cell lines that stably express optically active proteins whose degradations depend on different components of the UPS. Using these cell lines, we have established live cell degradation assays that employ the addition of MG132 to accumulate reporter, followed by a shift to CHX to monitor the rate of decay of the accumulated protein. Our approach can be broadly applied to any unstable proteins whose steady-state levels are barely detectable. Adapting our assays to an automated microscope platform enabled time-lapse analysis of the same plate and high speed data acquisition with high statistical reliability (*Z'*-factor ≥ 0.5 for all assays). At maximum throughput, the degradation assays as configured here can be used to screen 12,000 wells per day. Thus, even though monitoring reporter degradation involves more manipulation than monitoring accumulation, it is nevertheless suitable for HTS of sizable libraries. The dual-reporter cell lines and assay methods reported here can be used to rapidly categorize small molecule inhibitors and should be equally applicable to screen for and classify siRNAs, cDNAs, and peptides that have an impact on UPS function.

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