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Activity-Based Chemical Proteomics Accelerates Inhibitor Development for Deubiquitylating Enzymes

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SUMMARY

Converting lead compounds into drug candidates is a crucial step in drug development, requiring early assessment of potency, selectivity, and off-target effects. We have utilized activity-based chemical proteomics to determine the potency and selectivity of deubiquitylating enzyme (DUB) inhibitors in cell culture models. Importantly, we characterized the small molecule PR-619 as a broad-range DUB inhibitor, and P22077 as a USP7 inhibitor with potential for further development as a chemotherapeutic agent in cancer therapy. A striking accumulation of polyubiquitylated proteins was observed after both selective and general inhibition of cellular DUB activity without direct impairment of proteasomal proteolysis. The repertoire of ubiquitylated substrates was analyzed by tandem mass spectrometry, identifying distinct subsets for general or specific inhibition of DUBs. This enabled identification of previously unknown functional links between USP7 and enzymes involved in DNA repair.

INTRODUCTION

Successful drug development within the ubiquitin proteasome system (UPS) has enormous potential for the treatment of human disease, but faces considerable challenges (Bedford et al., 2011; Nalepa et al., 2006). These include the identification of focused lead compounds, the development of suitable assays for screening, and the availability of protein structures to aid rational drug design once promising hits have been identified. The need for selective inhibitors is exacerbated by the large number of deubiquitylating enzymes (DUBs) (Reyes-Turcu et al., 2009), ubiquitin conjugating enzymes (E2s), and ubiquitin ligases (E3s) (Hua and Vierstra, 2011; Rotin and Kumar, 2009) encoded by the human genome. Additional opportunities for pharmacological intervention are provided by the discovery of pathogen encoded factors that evolved to target the UPS of the host cell, representing attractive targets for treatments against infectious diseases (Edelmann and Kessler, 2008; Isaacson and Ploegh, 2009; Lindner, 2007).

Activity-based proteomics has contributed substantially to our understanding of the function of gene products (Cravatt et al., 2008; Evans and Cravatt, 2006). Molecular probes specific for the UPS allow tagging and detection of proteolytically active proteasome subunits (Bogyo et al., 1998; Ovaa et al., 2003), active deubiquitylating enzymes (DUBs) (Borodovsky et al., 2001, 2002) and ubiquitin-like (Ubl) specific proteases (reviewed in Hemelaar et al. [2004] and Ovaa [2007]). Members of the ligation machinery (E2s and E3s) are also detectable albeit with lower efficiency compared to DUBs (Love et al., 2009). In addition, the utility of activity-based ubiquitin-derived probes has been exploited for structural studies of ubiquitin-bound DUBs (Messick et al., 2008; Misaghi et al., 2005), in cell culture models of disease allowing the detection of the active DUB population (Sgorbissa et al., 2010), and for comparative tissue profiling (Altun et al., 2010; Ovaa et al., 2004).

The UPS plays fundamental roles in the regulation of protein turnover and function that are often altered in cancer progression, thereby providing entry points for the development of anti-tumor chemotherapeutics (Bedford et al., 2011; Nalepa et al., 2006). Targeting components of the ubiquitylation machinery may allow selective modulation of discrete substrates mediated by the specificity of Ub ligation (E2 and E3 enzymes) and deconjugation (DUBs) (Eldridge and O'Brien, 2010). Interference with either arm of this pathway should allow highly targeted pharmacological intervention, provided that compounds with sufficient selectivity can be identified (Marblestone et al., 2010; Nicholson et al., 2007; Sgorbissa et al., 2010).

Members of the DUB family that are known to contribute to neoplastic transformation include USP1 (Fanconi Anemia), USP2 (prostate cancer), DUB3 (stabilizing cyclin dependent kinase 25A), USP4 (adenocarcinoma), USP7/USP10 (stabilization of p53), USP9X (leukemias and myelomas), and BRCC36 (Hussain et al., 2009; Nijman et al., 2005; Pereg et al., 2010; Reyes-Turcu et al., 2009; Schwickart et al., 2010). In addition, mutations in the gene encoding the DUB CYLD can lead to the neoplastic condition Cylindromatosis, whereas other DUBs are expressed at lower levels in cancer including A20 (B cell and T cell lymphomas) and BAP1 (brain, lung, and testicular cancers) (Hussain et al., 2009).

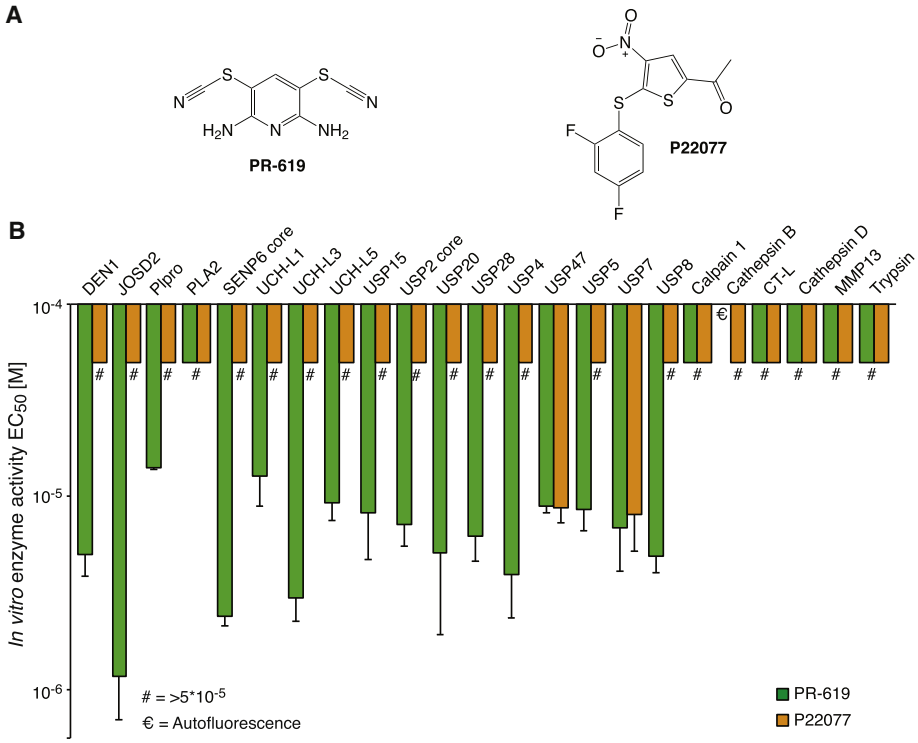


Figure 1. Structures of DUB Inhibitors PR-619 and P22077 and In Vitro DUB Inhibition Profiles

(A) Molecular structures of PR-619 and P22077.

(B) Inhibition profiles of PR-619 (green bars) and P22077 (orange bars) using a panel of DUBs, UBL-specific, cysteine, and other proteases as determined in the Ub-PLA_2 assay (Nicholson et al., 2008). USP5 and USP47 inhibition was assayed using Ub-EKL, and UCH-L1 and UCH-L3 inhibition with Ub-Rh110. Other cysteine proteases and other noncysteine proteases were assayed as described in the Experimental Procedures. €Indicates autofluorescence. #Represents EC_{50} values equal or larger than 5×10^{-5} [M]. Mean values and standard deviations from three or more independent experiments are shown. PLA_2, phospholipase A_2; USPs, ubiquitin-specific proteases; JOSD2, Josephin domain containing 2; UCH-Ls, ubiquitin C-terminal hydrolases; DEN1, deneddylase 1; PLpro, SARS-Co virus papain-like protease; SENP6, Sentrin-specific protease 6; CT-L, chymotryptic-like, 20S proteasome; MMP13, metalloproteinase 13. See also Figures S5 and S6 and Table S1.

USP7, also known as HAUSP, has been found to be critical in cancer progression due to its influence on the stability of the tumor suppressor p53 (Cheon and Baek, 2006; Colland, 2010; Nicholson et al., 2007). USP7 preferentially deubiquitylates the E3 ligase HDM2 and its binding partner HDMX as well as their substrate p53 (Brooks et al., 2007; Cummins and Vogelstein, 2004; Li et al., 2002, 2004; Meulmeester et al., 2005). Like most E3s, HDM2 has the capacity to auto-ubiquitylate and promote its own degradation. The cellular consequence of stabilizing HDM2 is the polyubiquitylation and subsequent degradation of p53. Thus inhibition of USP7 is predicted to destabilize HDM2 and stabilize p53. Additional substrates of USP7 have been reported including claspin, FOXO4, and PTEN (Faustrup et al., 2009; Song et al., 2008; van der Horst et al., 2006). Therefore USP7 exerts both p53-dependent and p53-independent effects on controlling cell proliferation and apoptosis, making USP7 an attractive target for pharmacological intervention in cancer (Colland et al., 2009; Goldenberg et al., 2008; Hussain et al., 2009; Nicholson et al., 2007; Nicholson and Suresh Kumar, 2011).

In an effort to identify small molecule inhibitors of USP7, two compounds that inhibit USP7 in the low μM range were identified using the Ub-CHOP reporter based screening assay (Goldenberg et al., 2008; Nicholson et al., 2008). The potency and selectivity of these molecules was evaluated in vitro and in living cells using an activity-based chemical proteomics approach. In this competition assay format, drug selectivity and potency was assessed in a straightforward manner using immunoblotting as readout. Further quantitative data were obtained by anti-HA immunoprecipitation after active site labeling with a HA-tagged DUB-specific molecular probe and quantitative mass spectrometry. In this way, P22077 was shown to inhibit USP7 functions in cells. In contrast, PR-619 was found to be a broad inhibitor of DUB activity. Moreover, our experiments demonstrate that DUB inhibitors with differing specificities induce the accumulation of polyubiquitylated proteins in cells without directly affecting proteasome activity. These results provide novel insights into DUB-associated proteins and pathways.

RESULTS

A robust in vitro assay that has been validated for high throughput screening applications (Ub-CHOP reporter system [Goldenberg et al., 2008; Nicholson et al., 2008]) was employed to sample a small molecule diversity based library for modulators of USP7 activity. Two of the confirmed hits from this screen were PR-619 (2,6-diaminopyridine-3,5-bis(thiocyanate)) (Figure 1A)

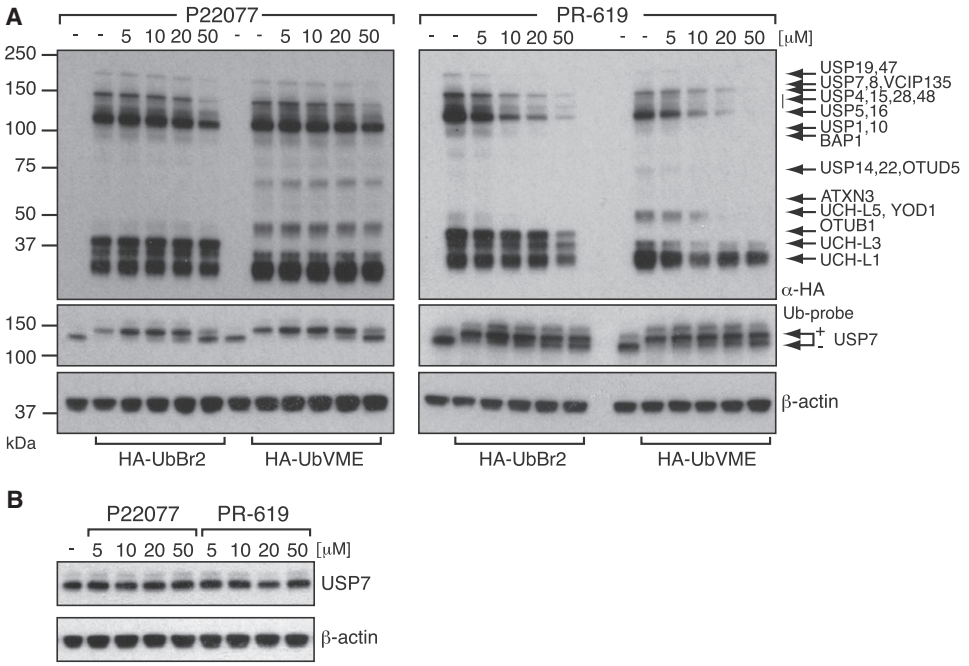


Figure 2. Small Molecule Inhibitors Affect DUBs in Living Cells

The inhibitors PR-619 and P22077 were incubated with HEK293T cells for 6 hr at concentrations of 5, 10, 20, or 50 μM. DMSO (0.1%) was used in control lanes. (A) Crude cell extracts were labeled with HAUbVME or HAUbBr2 for 30 min at 37°C prior to separation by 4%–12% Bis-tris SDS-PAGE and immunoblotting with HA, USP7, and β-actin (loading control) antibodies. + and – indicates USP7 in a free form (–) or labeled with HA-UbVME or HA-UbBr2 (+), respectively. (B) Input loading controls of extracts prepared from inhibitor treated cells showing equal USP7 and β-actin levels by immunoblotting. See also Figures S1, S5, and S6.

and P5091. Initial biochemical characterization revealed that P5091 selectively inhibited USP7 relative to other DUBs and other families of proteases, and subsequent medicinal chemistry optimization generated additional analogs including P22077 (1-(5-((2,4-difluorophenyl)thio)-4-nitrothiophen-2-yl)ethanone) (Figure 1A) (Tian et al., 2011). The inhibitory activities of P22077 and PR-619 were compared in vitro against a panel of DUBs, cysteine proteases, and other families of proteolytic enzymes. Data from these studies demonstrate that P22077 inhibits USP7 and the closely related DUB USP47 (Figure 1B; see Table S1 available online). In contrast, PR-619 exhibits a broader inhibitory profile targeting multiple DUBs, but with limited activity against other families of proteases, including representative examples of other families of cysteine proteases (Figure 1B; Table S1).

To address the effects of these compounds in a cellular context, DUB inhibitory capacities of PR-619 and P22077 were tested in crude cell extracts. To this end, a competition assay was performed against the DUB active site probes HA-UbBr2 and HA-UbVME (Borodovsky et al., 2002; Hemelaar et al., 2004). Human embryo kidney (HEK293T) cell extracts were incubated with increasing concentrations of PR-619 or P22077 followed by labeling with HA-UbBr2 or HA-UbVME to profile residual DUB activity (Figure S1). The identification of DUBs by immunoblotting was based on molecular weights of known DUBs, a comparison with labeling patterns from previous studies (Borodovsky et al., 2002; Hemelaar et al., 2004) and identification by mass spectrometry as shown below. Compound PR-619 inhibited the majority of DUBs labeled by HA-UbBr2 and HA-UbVME at a concentration of 20 μM, and to a lesser extent at 5 μM (Figure S1A). In contrast, compound P22077 inhibited a much smaller subset of DUBs at a concentration of 15–45 μM (Figure S1B). This indicated overlapping binding of the two compounds and the ubiquitin-based probes, and a greater selectivity of P22077 over PR-619 in targeting DUBs. Both compounds inhibited probe binding to USP7 in the low micromolar range.

Having established the inhibition profiles of cellular DUBs upon treatment with PR-619 and P22077 in cell lysates, experiments on living cells were conducted. Concentration ranges for the inhibitors were determined by cytotoxicity assays that were performed in HCT-116 colorectal cancer and HEK293T cells. Data from these studies demonstrated that P22077 and PR-619 induce (tumor) cell death with EC_{50} values in the low micromolar range (Figures S2A and S2B).

Cell permeability and the potency of DUB inhibition in living cells were addressed in another set of experiments. This involved incubation of HEK293T cells with PR-619 or P22077, followed by lysis, labeling with HA-UbVME or HA-UbBr2 and immunoblotting analysis (Figure 2). PR-619 interfered with probe labeling at concentrations of 5 μM and higher (Figure 2A). As observed previously, P22077 at 20 μM did not have any notable effect on the overall active site profiling with the HA-tagged probes and affected only a subset of DUBs at 50 μM. However, USP7 labeling was partially inhibited at 20 μM P22077, and complete inhibition was observed at 50 μM or higher concentrations.

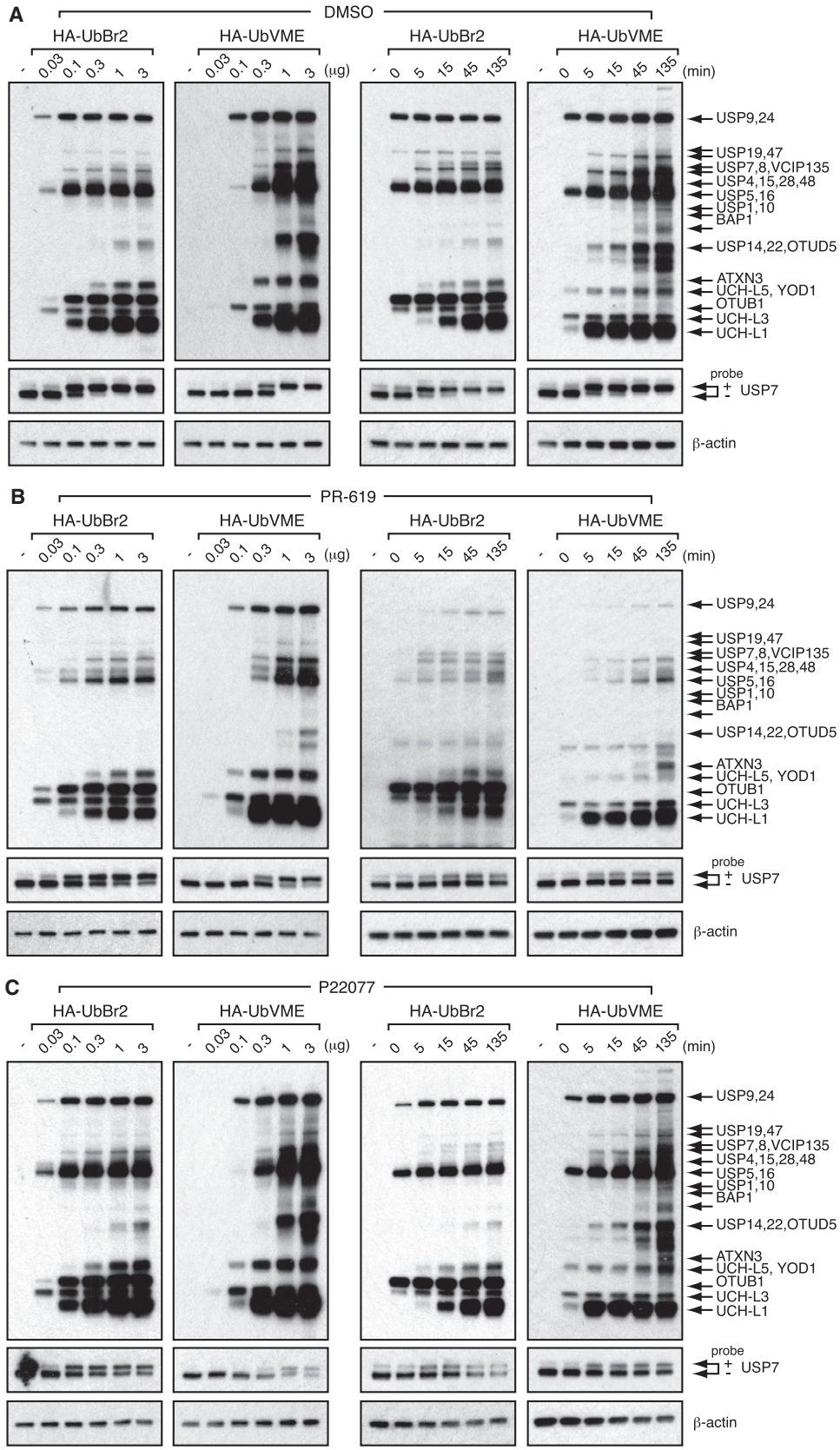


Figure 2B shows USP7 protein input levels at all inhibitor concentrations tested. PR-619 also inhibited labeling of USP7 by the probes, but at the same time targeted many other DUBs within the same concentration range, consistent with its broader inhibitory profile.

Exposure of inhibitor—treated cell lysates to the HA-Ub—probes, which typically bind covalently and irreversibly to active DUBs, may result not only in labeling of residual DUB activity but also in gradual replacement of the reversible inhibitors from the enzyme's active sites. In addition, the HA-Ub probes may preferentially label a subset of DUBs that may also modulate such a read out. These parameters may influence the inhibition profiles obtained from such assays. To address the HA-Ub probe's preferences for DUBs, HEK293T cell extracts were incubated with different amounts of HA-UbBr2 or HA-UbVME, or at a fixed concentration for different times (Figure 3A). Both probes appear to label UCH-L3, USP5/16, and USP9/24 most efficiently, and HA-UbBr2 preferentially targets OTUB1. However, upon prolonged incubation, the labeling profile becomes more complex (Figure 3A). To test whether under these circumstances reversibility with the small compounds may occur, we incubated PR-619 and P22077 with HEK293T cell lysates prior to labeling with different concentrations of HA-UbVME or HA-UbBr2 for various length of time (Figures 3B and 3C). In both cases, inhibition by the small molecule compounds persisted even after labeling with the Ub-probes for >2 hr (Figures 3B and 3C). The effect of inhibition varied between the different DUBs due to different affinities of the compounds but also the Ub-probes themselves as described for Figure 3A. Furthermore, little to no displacement was observed, in particular for USP7 and P22077 under the experimental conditions used (Figure 3C).

Although profiling experiments using ubiquitin-based active site-directed probes gave an insight into the DUBs targeted by P22077 and PR-619 in living cells, immunoblotting did not provide sufficient information to distinguish between all the different USPs, as many of them have similar molecular weights, in particular in the 100–150 kDa range. We therefore developed an activity-based quantitative proteomics approach to overcome this problem. In this method, HEK293T cells were treated with DMSO or 25 μM concentrations of PR-619 or P22077 for 6 hr prior to cell lysis and labeling with HA-UbBr2 or HA-UbVME (Figure 4A). DUBs were thereby labeled differentially depending on the inhibition profile of the tested inhibitor. DUB-HA-Ub-probe adducts were immunoprecipitated with anti-HA beads and eluted material subjected to in-solution trypsin digestion. The samples were then analyzed in triplicate by label-free quantitative mass spectrometry (MS) using nano-UPLC-MS/MS, resulting in a differential display of active DUBs in inhibitor treated relative to control cells. Two independent experiments were conducted, and we identified 49 DUBs, among which 34 were pulled down using the HA-UbBr2 probe and 48 with the HA-UbVME probe (33 DUBs were identified with both probes (Figure 4B; Table S2; data not shown). For 25 of the DUBs identified, relative quantitative information was obtained, indicating different degrees of specificity for the inhibitors PR-619 and P22077 (Figure 4B). As expected, PR-619 showed a much broader inhibitory profile as compared to P22077, which mainly targeted USP7, but also USP47 when tested at 25 μM (Figure 4B). The differential targeting of DUBs between the two inhibitors is also reflected in the immunoblotting profiles after enzyme capture with the HA-UbVME probe, which was less efficient in the presence of PR619 as compared to DMSO or P22077 (Figure 4C). Furthermore, the inhibition profiles observed by quantitative mass spectrometry were further validated for a subset of DUBs by immunoblotting with antibodies specific for USP9, USP47, USP7, USP15, USP5, USP14, OTUB1, UCH-L3, and UCH-L1 (Figure 5). Inhibition of a particular DUB was indicated by interfering with the mass shift provoked by labeling with HA-UbBr2 or HA-UbVME (indicated with + and −, Figure 5). USP7 labeling by HA-UbBr2 and HA-UbVME was partially impaired in the presence of PR-619 and P22077, whereas all other DUBs tested were essentially unaffected by 25 μM P22077 with the exception of USP47 when labeled with HA-UbBr2. Labeling of all DUBs by both probes was affected partially by 25 μM and fully by 50 μM PR-619. In addition to the ones validated by immunoblotting in Figure 5, MS analysis revealed that PR-619 also interfered with labeling of USP1, 4, 8, 10, 16, 19, 22, 24, 28, 48, VCIP135, OTUD5, BAP1, ATXN3, YOD1, and UCH-L5 (Figure 4B).

Having demonstrated the DUB inhibitory profiles of PR-619 and P22077 in living cells, we explored the effect of these inhibitors on the homeostasis of polyubiquitylated material. This may provide an entry point for identifying substrate candidates for DUBs targeted by these compounds, and offers valuable information on the cellular effects of these inhibitors. To this end, HEK293T cells were treated with PR-619 or P22077 for 0.5 up to 20 hr or at different doses followed by cell lysis and anti-ubiquitin immunoblotting. Interestingly, treatment of cells with PR-619 as well as P22077 led to the accumulation of polyubiquitylated material in a dose- and time-dependent fashion (Figures 6A and 6B). In the case of PR-619, incubation times of >6 hr or >50 μM led to loss of cell material likely due to toxicity (Figure 6B). This effect was not due to direct inhibition of the proteolytic activity of the proteasome as shown by competitive labeling of the active proteasome β-subunits and in vitro enzyme activity assays (Figure 1B; Figure S3, and Table S1). To shed further light on the nature and type of ubiquitylated proteins that accumulated upon exposure to inhibitors, polyubiquitylated material was isolated from cells using GST-TUBEs (tetraubiquitin binding entities) (Figure 6C). We used TUBE1 and TUBE2 that possess a high binding affinity for K^{48}- and K^{63}-polyubiquitin chains (Hjerpe et al., 2009). A varying amount of ubiquitylated protein material was observed when cells were exposed to 0.1% DMSO, 25 μM PR-619, or 25 μM P22077, indicating differences in ubiquitylated proteins that were accumulating. We subjected eluted material from TUBE1 and TUBE2 affinity

Figure 3. Dynamics and Specificity of Activity-Based DUB Profiling in Cells

HEK293T cells were treated either with (A) DMSO, (B) 25 μM PR-619, or (C) 25 μM P22077 for 6 hr at 37°C. Crude extracts were prepared and incubated with increasing concentrations of HA-UbBr2 or HA-UbVME active site probes (left two panels) or 2 μg of probe for the indicated times (right two panels). Samples were separated by 4%–12% Bis-tris SDS-PAGE and analyzed by immunoblotting with HA, USP7, and β-actin (loading control) antibodies. See also Figures S1, S5, and S6.

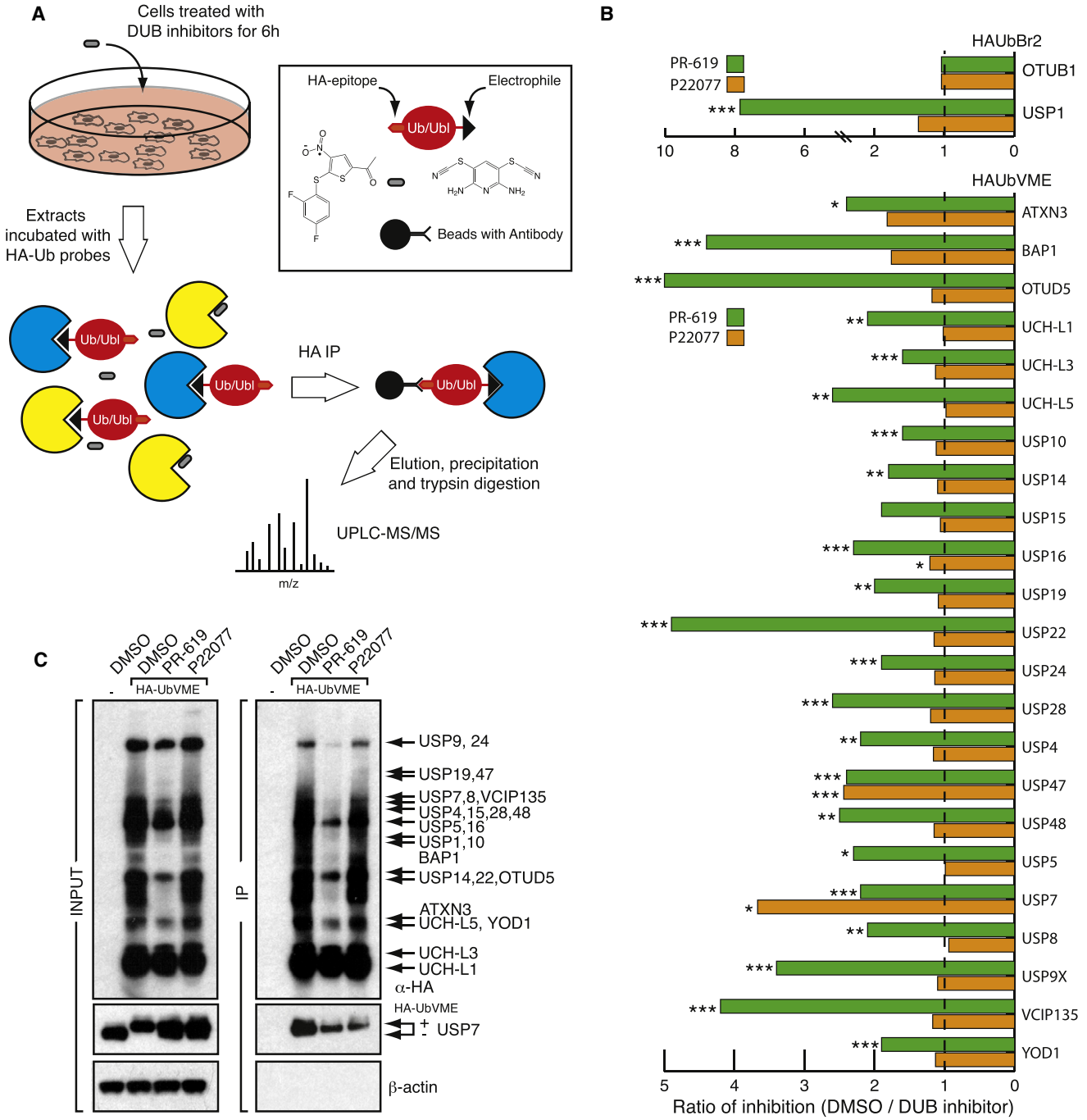


Figure 4. DUB Inhibition Profile in Living Cells Revealed by Activity-Based Quantitative Mass Spectrometry

(A) HEK293T cells were treated with 25 μM PR-619 or 25 μM P22077 for 6 hr. Crude extracts were incubated with either HAUbVME or with HAUbBr2 followed by anti-HA immunoprecipitation. Eluted material was digested with trypsin and subjected to label-free quantitative mass spectrometry (UPLC-MS/MS) analysis.

(B) Inhibition profiles of 25 DUBs identified by tandem mass spectrometry (UPLC-MS/MS). Relative abundance ratios of inhibition based on DUBs isolated from cells treated with PR-619 (green bars) or P22077 (orange bars) as compared to controls. Top panel: unique DUBs isolated using the HA-UbBr2 probe. Bottom panel: DUBs isolated using the HA-UbVME probe. The abundance of each DUB is based on the ion intensities of tryptic peptides matching a unique DUB protein sequence identified in triplicate analytical runs. Analysis of variance (ANOVA) was used to calculate the statistical significance of the observed changes: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (see Supplemental Information). One of two independent experiments is shown.

(C) As described above, cellular DUBs were isolated by HA-UbVME labeling and anti-HA immunoprecipitation. As a control for the MS experiment (B), input (left panel), and anti-HA immunoprecipitated material were separated by 4%–12% Bis-tris SDS-PAGE and analyzed by immunoblotting with HA, USP7, and β-actin (loading control) antibodies. See also Figure S5 and Table S2.

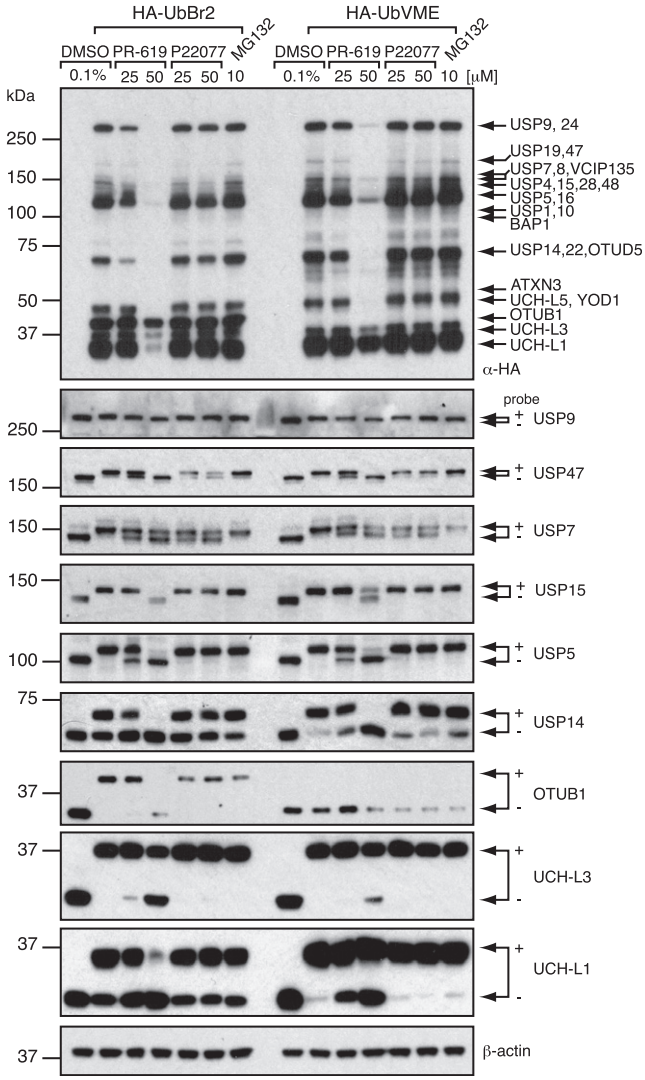


Figure 5. Inhibition of Selected DUBs by P22077 and PR-619 in Living Cells Detected by Activity-Profiling and Immunoblotting

HEK293T cells were treated with DMSO (lane 2), PR-619 (25 and 50 μM, lanes 3/4), P22077 (25 and 50 μM, lanes 5/6), or MG-132 (10 μM, lane 7) for 6 hr, followed by cell lysis and labeling with HAUbVME or HAUbBr2 (lane 1 is lysate alone). Samples were separated by 4%–12% Bis-Tris SDS-PAGE and analyzed using HA-, USP5-, USP7-, USP9-, -USP14, -USP15, -USP47, -OTUB1, -UCH-L1, and -UCH-L3 antibodies. β-actin was used as a loading control. See also Figures S5 and S6.

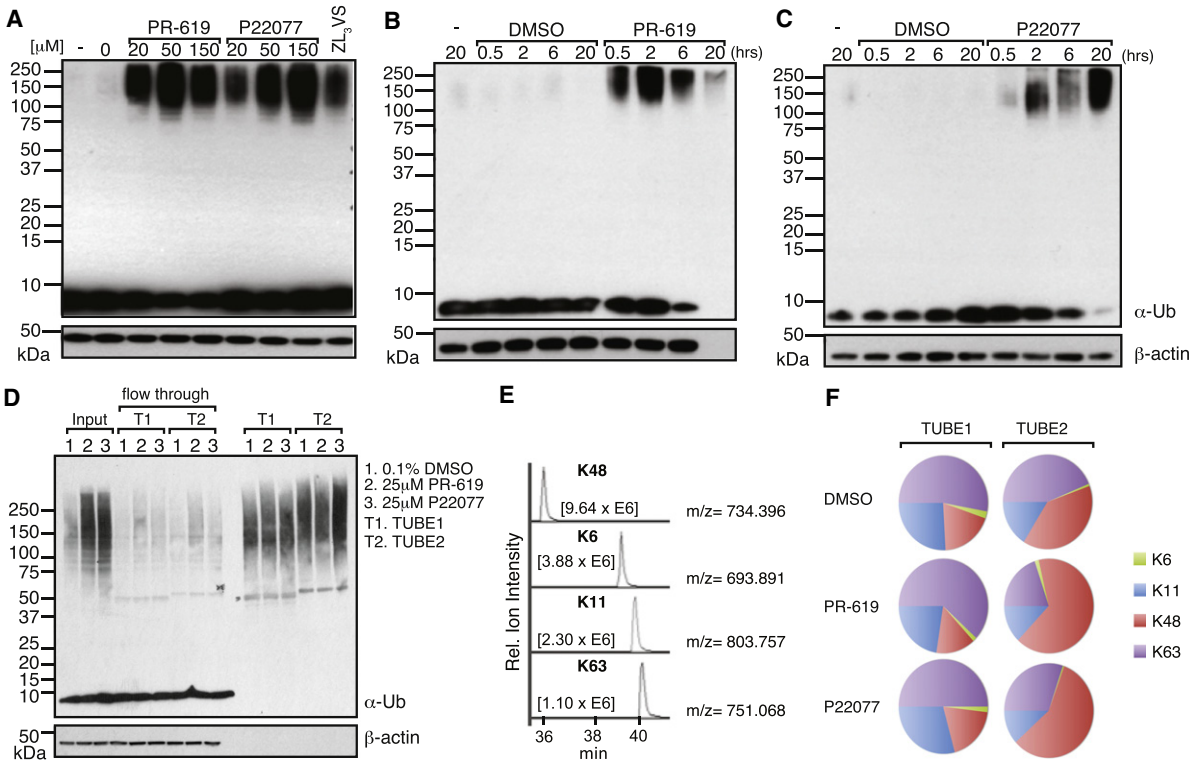
purification to in-solution trypsin digestion and subsequent analysis by tandem mass spectrometry (Figure 6G; Table S3). This revealed relative quantitative information on 232 proteins (Figure 6G; Table S3). Ubiquitin was found not only as lys^{48}-linked, but also as lys^{63}, lys^{11} and lys^{6}-linked poly-Ub chains in untreated, but also cells treated with PR-619 and P22077 (Figures 6D–6F). No evidence for the presence of lys^{27}-, lys^{29}-, and lys^{33}-linked poly Ub-chains was found. Isotopically labeled peptide standards representing Ub derived tryptic fragments with lys(gly-gly) tags were used to quantitate the degree of Ub-linkages detected in cell extracts (Figure 6E, see also Supplemental Experimental Procedures). Interestingly, PR-619 inhibition resulted in an accumulation of K^{48}- and K^{63}-, whereas P22077 exposure led to enrichment of mainly K^{48}-linked poly-Ub chains (Figure 6F). Components of the 26S proteasome complex were also accumulated when cells were exposed to either inhibitor (Figure 6G). P22077 inhibition exhibited changes in ubiquitylated protein levels that were distinct from the broad specificity inhibitor. Proteins observed at differential abundance in control as compared to P22077-treated cells could represent either direct or indirect substrates of the DUBs targeted by this inhibitor (mostly USP7 and probably USP47 under these circumstances).

To provide experimental evidence for this, we examined the fate of the well known USP7 target, HDM2, upon pharmacological inhibition of USP7 with P22077 (Figure 7). HDM2 levels decreased initially after 2 hr of treatment as expected when not deubiquitylated by USP7 (Figure 7A; Figures S4A and S4B), in line with independent studies in $USP7^{-/-}$ cells (Cummins et al., 2004; Meulmeester et al., 2005). p53 protein and its transcriptional target p21 accumulated after 8 hr of treatment (Figure 7A; Figures S4A and S4B), consistent with previous studies in which USP7 levels were ablated (Li et al., 2004). The increase in p53 protein induces a feedback mechanism that stimulates additional HDM2 (Vogelstein et al., 2000). Subsequently, p53 levels drop again (likely due to the elevated HDM2).

As an additional confirmation that P22077 is directly inhibiting USP7 we also determined the ability of P22077 to destabilize claspin, a scaffolding protein required for Ataxia telangiectasia and Rad3 related (ATR) mediated phosphorylation of the checkpoint kinase Chk1 and that is subject to ubiquitin dependent proteasomal degradation (Faustrup et al., 2009; Kumagai and Dunphy, 2000; Mailand et al., 2006). In agreement with published reports, P22077 treatment of U2OS cells during release from G1/S arrest induced with hydroxyurea resulted in a dose-dependent loss of claspin protein and a concomitant decrease in phospho Serine 317 Chk1 (Figure 7B). Furthermore, quantitative MS suggested the E3 ubiquitin ligase components RBX1, DCAF7, DCAF11, and the DNA damage binding protein 1 (DDB1) to be reduced upon cellular treatment with P22077 (Figure 6G; Table S3). A reduction in DDB1 protein levels was confirmed by immunoblotting (Figure 7C). To test whether this was due to incapacitation of USP7 or other DUBs, USP7, USP47, and USP15 were knocked down and the levels of DDB1 assessed under these conditions. Consistent with the P22077 data, DDB1 levels were only reduced when USP7 was knocked down (Figures 7D and 7E).

DISCUSSION

Recent studies have uncovered a range of small molecules with inhibitory activity against DUBs such as UCH-L1 (Liu et al., 2003), PLpro (Ratia et al., 2008), and USP7 (Colland et al., 2009; Tian et al., 2011). Testing specificity and selectivity of small molecular compounds toward DUBs is challenging, and is in most cases performed in vitro using a panel of recombinant proteolytic enzymes in enzymatic assays. Alternatively, genetically engineered substrates for enzymes tagged with fluorescent reporters can be generated recombinantly or expressed in cells and used as readouts for specific enzyme (DUB) activities (Nicholson et al., 2008; Shanmugham and Ovaa, 2008). However, this only



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Differentially ubiquitinated proteins present in cells treated with DUB inhibitors identified by LC-MS/MS

Protein Family	*Protein name*	*Swissprot [Acc. Nr.]*	*PR-619/Ctrl Ratio*[c]	*P22077/Ctrl Ratio*[c]	*ANOVA [P]*[d]	*Isolation Method*
26S proteasome complex	20S, 19S subunits	12 identified[a]	3.2-14.5	1.7-5.6		TUBE1/2[b]
Ub conjugation/ deconjugation	E3 UBR5	O95071	4.3	1.5	<0.01	TUBE2
	E3 AMFR	Q9UKV5	3.7	3.1	0.02	TUBE2
	E3 CHIP (STUB1)	Q9UNE7	14	0.6	0.01	TUBE1
	E3 HECTD3	Q5T447	0	0	<0.01	TUBE1
	E3 MKRN2	Q9H000	34.5	0.4	0.05	TUBE2
	E3 RNF126	Q9BY78	5.9	1.5	0.01	TUBE1
	E3 RNF25	Q96BH1	8	1.9	<0.01	TUBE2
	E3 RNF214	Q8ND24	>100	>100	<0.01	TUBE1
	E3 RBX1	P62877	0.6	0.5	<0.01	TUBE2
	E3 Cullin 1	Q13616	0.8	0.6	0.02	TUBE2
	E3 Cullin 3	Q13618	5.8	1.5	<0.01	TUBE2
	UBE2Q2P1 (E2 enzyme-like)	Q6ZUW4	0.6	1.9		TUBE1
Ubiquitin related	SUMO3	P55854	5.8	0.2	<0.01	TUBE2
Transcription / DNA repair	DCAF7 (DDB1 and CUL4 assoc. factor 7)	P61962	8.9	0.6	<0.01	TUBE1
	DCAF11	Q8TEB1	0.1	0.5	<0.01	TUBE2
	DCAF11	Q8TEB1	0.6	0.7	0.05	TUBE1
	DDB1 (DNA damage bdg protein 1)	Q16531	0.3	0.5	<0.01	TUBE2
	XRCC5 (Ku80)	P13010	0.2	0.5	<0.01	TUBE2
	XRCC6 (Ku70)	P12956	0.3	0.5	<0.01	TUBE2
	PARP1	P09874	0.4	0.4	<0.01	TUBE2
	TP53	P04637	10.6	1.1	0.01	TUBE1

Figure 6. General and Specific DUB Inhibition Lead to Accumulation of Polyubiquitylated Proteins

(A) HEK293T cells were treated with PR-619 or P22077 for 2 hr at the indicated concentrations or 10 μM Z-Leu-Leu-Leu-vinyl sulfone (ZL_3VS) as a control for proteasome inhibition, and cell lysates analyzed by 4%–12% bis-tris SDS-PAGE and anti-ubiquitin immunoblotting. β-actin was used as a loading control.

(B and C) HEK293T cells were treated with 0.1% DMSO, (B) 50 μM PR-619, or (C) 50 μM P22077 for the indicated length of time and analyzed as indicated above.

(D) HEK293T cells were treated for 6 hr with 0.1% DMSO (lane 1), 25 μM PR-619 (lane 2), or 25 μM P22077 (lane 3) followed by cell lysis and coimmunoprecipitation of polyubiquitylated material using GST-tagged tetraubiquitin binding entities 1 (TUBE1/T1) or TUBE2 (T2). Input, flow-through and TUBE1 and 2 eluted material was analyzed by 4%–12% bis-tris SDS-PAGE and anti-ubiquitin immunoblotting. β-actin was used as a loading control.

(E and F) Analysis of poly-Ub linkages by mass spectrometry. One hundred femtomoles of K^{48}-, K^{6}-, K^{11}-, and K^{63}-linked Gly-Gly containing standard peptides were separated by UPLC and analyzed by MS. Ion peak intensities were used as correction factors to calculate the percentage [%] of different poly-Ub linkages enriched with TUBE1 and 2 (F) and after treatment either with DMSO, PR-619, or P22077 (see also Supplemental Information).

provides information about enzymes that are tested, and with at least 550 proteases encoded by the human genome that represents a challenging task (Puente et al., 2003). Furthermore, a large proportion of the DUB family exhibit isopeptidase activity, potentially reducing the usefulness of genetically encoded substrates.

We utilized the ubiquitin-based active site probes HA-UbVME and HA-UbBr2 that were previously demonstrated to target a broad range of DUBs with no detectable cross-reactivity to other proteolytic enzymes (Borodovsky et al., 2002; Hemelaar et al., 2004). This can be used as an enrichment step to isolate active DUBs following previous inhibitor treatment of cells or crude extracts, providing a "competition profile" of the inhibitors that can be visualized by immunoblotting or characterized by quantitative tandem mass spectrometry. The latter allowed measuring 49 DUBs by UPLC-MS/MS, representing half of all currently known human DUBs (Figure 4; Table S2). These results indicated the broad inhibitory nature of PR-619 and the more selective properties of P22077 toward USP7, and these are particularly relevant as this is within the physiological context of endogenous DUBs (Figures 4 and 5). The best selectivity of P22077 was observed when cells were treated with 20 μM P22077, with a cross-reactive effect against USP47 (Figure 4B and in part in Figure 5). This was consistent with the inhibition profiles observed when tested against recombinant enzymes (Figure 1B; Table S1). USP7 and USP47 share considerable homology (Parsons et al., 2011) and may therefore be susceptible to inhibition by the same small molecule. UCH-L5 was affected when cell extracts were treated with P22077 (Figure S1), although this was not the case in cell based experiments (Figures 2, 3, 4, and 5) nor with recombinant enzyme (Figure 1B). This discrepancy may result from an association/dissociation of UCH-L5 from the proteasome complex that may affect activity, which might be altered upon the preparation of cell extracts. Although selectivity against USP7 may be required for a maximal therapeutic effect in anti-cancer treatments, rigorous testing will reveal whether a certain degree of cross-reactivity to other DUBs could be beneficial or cause unwanted side-effects.

To gain further insights into how selective inhibition of DUBs in cells can affect intracellular processes, a proteomics analysis of polyubiquitylated material that accumulated after inhibitor treatment of HEK293T cells was performed. The use of tetraubiquitin binding entities (TUBEs 1and 2) enriched for mainly K^{48}- and K^{63}-linked polyubiquitylated material as detected by tandem mass spectrometry (Hjerpe et al., 2009). Treatment with both inhibitors led to the accumulation of mainly K^{48}-linked poly-Ub material (Figures 6E and 6F). TUBE1 and 2 both have nanomolar affinities for K^{48}- and K^{63}- linked poly-Ub chains, so it may be feasible that other chain linkages were isolated indirectly as part of branched Ub structures (Kim et al., 2007). Broad DUB inhibition by PR-619 as well as interference with mainly USP7 by P22077 resulted in an accumulation of 26S proteasome complexes (Figure 6G; Table S3), but did not directly block proteolytic activity of the proteasome at the concentrations employed (Figure 1B; Figure S3, and Table S1). However, a general accumulation of high molecular weight polyubiquitylated material was observed (Figure 6). This observation may be explained by a potential "overload" of proteasomal proteolysis capacity with accumulated polyubiquitylated material binding to subunits of the 19S complex, resulting in co-isolating 26S proteasomes using TUBEs. Despite the more selective nature of P22077 relative to PR-619, we observed an accumulation of polyubiquitylated material when cells were treated with either of these inhibitors (Figure 6). To address whether the incapacitation of a single DUB, such as USP7, can lead to poly-Ub accumulation, we used siRNA knockdown experiments. Interestingly, we detected no noticeable increase in poly- and monoubiquitin material (data not shown). The discrepancy between the pharmacological inhibition and siRNA based reduced expression may result from the fact that a knockdown leads to the disappearance of the entire protein, whereas the inhibitor inactivates the enzyme. Also, a knockdown occurs in a time frame of 48–72 hr, leading to compensatory mechanisms for the turnover of poly-Ub material not seen upon acute inhibitor treatment (2–6 hr). Alternatively, P22077 may also inhibit other DUBs not assayed in this study. Inhibition with PR-619 and P22077 led to the enrichment of a number of E3 ubiquitin ligases/ligase components including UBR5, AMFR, RNF126, RNF25, RNF214, and Cullin 3, whereas the E3 ligases/ligase components HECTD3, RBX1, and Cullin 1 were reduced (Figure 6G; Table S3). The ubiquitylation and turnover of potential substrates (such as E3 ligases) or subsequent processes dependent on DUBs are expected to be increased upon DUB inhibition, leading to their disappearance when compared to untreated controls. The occurrence of both accumulation and reduction in protein levels is currently unexplained, but could be due to a selective degradation of short-lived, and an accumulation of long-lived (ubiquitylated) proteins that may be aggravated upon DUB inhibition. Alternatively, inhibition of DUBs involved in the stabilization of E3 ligases can lead to indirect accelerated turn-over of substrates (including other E3 ligases) or vice versa, thereby complicating the picture of proteins that are affected by the pharmacological intervention of deubiquitylation. Consistent with the notion that P22077 predominantly inhibits USP7, we noted altered levels of HDM2, p53 and p21 upon treatment of cells with P22077 (Figure 7A; Table S3). The initial decrease in HDM2 after 2 hr followed by an increase in p53 and its transcriptional target p21 are consistent with previous reports (Cummins and Vogelstein, 2004; Li et al., 2004). The increase in HDM2 after 8 hr of treatment is likely due to the feedback loop between p53 and HDM2 (Vogelstein et al., 2000). Also, claspin, a scaffolding protein stabilized by USP7 that is involved in regulating the Chk1 kinase activated during the DNA damage response (Faustrup et al., 2009), is destabilized upon cellular treatment with P22077 (Figure 7B), providing further confidence that USP7 is one of the main pharmacological targets in cells.

(G) Differentially ubiquitylated proteins present in cells treated with DUB inhibitors identified by tandem mass spectrometry. [a]P35998,P62333,O00487, Q16186,O00231,O00232,P28074, A8K3Z3,Q99436,O75832,O75832, O43242, $p < 0.01$. [b]TUBE: tetra-ubiquitin binding entities; TUBE1: UBA derived from RAD23; TUBE2: UBA derived from Ubiquilin (Hjerpe et al., 2009). [c]Ratio determined using LC-Progenesis software, based on the mass peak ion intensities of peptides assigned to proteins (see Supplemental Information). [d]Analysis of variance (ANOVA) for assessing the significance of changes in ratio ($p < 0.05$). See also Figures S3, S5 and S6 and Table S3.

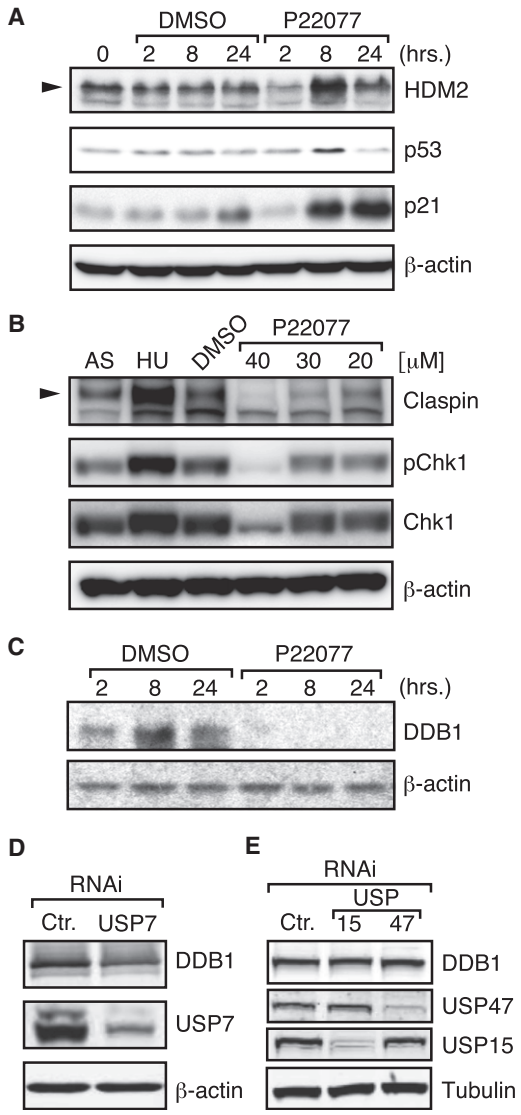

Figure 7. P22077 Affects USP7 Targets and DDB1 in Cells

(A) Treatment of HCT116 cells with 25 μM of the selective inhibitor P22077, but not with 0.1% DMSO, leads to increased levels of HDM2, p53 and p21. One representative out of three independent experiments is shown. See also Figure S4.

(B) U2OS cells were treated with hydroxyurea (HU) to synchronize them, prior to incubation with the indicated concentrations of P22077 for 8 hr (AS, asynchronous cells). Cells were lysed and extracts separated by SDS-PAGE followed by immunoblotting analysis using claspin, phospho-317-Chk1, Chk1, and β-actin antibodies.

(C) HEK293T cells were treated with DMSO or 25 μM P22077 for the indicated times. Crude cell extracts were then separated by SDS-PAGE and analyzed by anti-DDB1 immunoblotting.

(D and E) HEK293T cells were transfected with siRNAi specific for USP7, USP15, or USP47 for 72 hr as described in Supplemental Experimental Procedures. Crude cell extracts were separated by SDS-PAGE and analyzed by immunoblotting using DDB1, USP7, USP15, USP47, and β-actin antibodies. See also Figures S3, S4, S5, and S6.

Furthermore, we observed the absence of the DNA damage protein DDB1, RBX1, DCAF7 and DCAF11, all of which are subunits of E3 ubiquitin ligases (Hu et al., 2004), indicating that USP7 may be involved in altering the stability of such complexes. As a validation of the proteomic screen data we demonstrated that USP7, but not USP15 or USP47 knockdown led to a decrease in DDB1 protein levels (Figure 7C). A connection between USP7 with the DNA damage response has been described, possibly via histone deubiquitylation (Khoronenkova et al., 2011), and is further strengthened by its ability to stabilize claspin, a protein necessary for Chk1 mediated cell cycle arrest following DNA damage (Faustrup et al., 2009; Scrima et al., 2011). Potentially, these findings could be exploited clinically by developing a synergistic therapeutic approach consisting of inhibition of USP7 to ablate the G2/M checkpoint in combination with a genotoxic agent, resulting in more efficient cancer therapies.

SIGNIFICANCE

Novel approaches to screen small molecule inhibitors in their natural environment within the cell are of major importance to elucidate inhibitor selectivity and specificity. Here we demonstrate a powerful chemistry-based functional proteomics and mass spectrometry method for screening DUB inhibition in living cells. Using an inhibitor with selectivity for USP7, we describe a possible link between this DUB and aspects of the DNA damage response, which may have implications for novel combinatorial anticancer therapies. In general, this novel approach permits the determination of inhibitor profiles against endogenous DUB levels in cells/tissues under healthy and pathological circumstances, and can be adopted for the drug screening processes in biotech, pharma, and academia at an advanced stage to further select promising lead compounds. Additional advantages include the ability to confirm the cellular permeability of inhibitors and examine compounds that cannot be tested for enzyme inhibition due to autofluorescent properties. Cell-based assays with an active-site probe readout for direct measurement can also be applied to other enzyme classes, and can be multiplexed using several probes with different tags, which may uncover a wider range of target leads and more than one enzyme species affected by the compound. Specific and promising small molecules discovered by a combination of high throughput screening and cell-based MS profiling as described in this study can be used as lead compounds for disease treatment, but also as research tools to understand the underlying mechanisms of the biology of targeted enzymes.

EXPERIMENTAL PROCEDURES

Cell Lines and Reagents

HEK293T and HeLa cells were grown in DMEM medium supplemented with 10% FCS, 1% penicillin, and 1% Glutamax at 37°C in the presence of 5% CO_2. HCT116 and U2OS cells were grown in DMEM, 10% FBS, 1% P/S, and 2 mM glutamine (5% CO_2). Chemicals were purchased from Sigma-Aldrich unless indicated otherwise. The following antibodies were used: anti-OTUB1 (Edelmann et al., 2010), anti-USP5 (Lifesensors, PA), anti-USP7 (Bethyl Laboratories, TX), anti-USP9, anti-USP14, anti-UCH-L1, anti-UCH-L3, anti-HDM2, and anti-USP47 (Santa Cruz Biotechnology, CA), anti-β actin,-tubulin (Sigma), anti-USP15 (Bethyl Laboratories), anti-p53 (Calbiochem), anti-p21 (Cell Signaling), anti-Claspin (Bethyl), phospho-Chk1Ser317, and anti-Chk1 (Cell Signaling).

Characterization of the Inhibitors PR-619 and P22077

The synthesis and analysis of PR-619 (Beer et al., 2002) and P22077 are described in detail in Supplemental Experimental Procedures and Figures S5 and S6.

In Vitro Enzyme Assays

Recombinant full length USP7, USP2 core, USP5, JOSD2, DEN1, PLpro core, and SENP2 catalytic core were generated as previously described (Nicholson et al., 2008). Amino terminal His_6 tagged USP4, USP8, USP28, UCH-L1, UCH-L3, UCH-L5, and MMP13 were expressed in *Escherichia coli*. N-terminal His_6 tagged USP15, USP20, and USP47 were expressed in Sf9 cells. All the recombinant proteins were purified by chromatography. Amino terminal tagged His_6 Ub-PLA_2 (Ub-CHOP), SUMO3-PLA_2 (SUMO3-CHOP), ISG15-PLA_2 (ISG15-CHOP), NEDD8-PLA_2 (NEDD8-CHOP), Ub-EK_L (Ub-CHOP2), and free catalytically active PLA_2 were prepared as described (Nicholson et al., 2008; Tian et al., 2011).

Activity Based Profiling of DUB Inhibitors in Crude Extracts and Living Cells

The labeling of endogenous active DUBs in crude cell extracts using the active site molecular probes HA-UbVME and HA-UbBr2 was performed essentially as described (Borodovsky et al., 2002) and further described in the Supplemental Information.

Isolation of Ubiquitylated Proteins

For the isolation of polyubiquitylated material from control or cells treated with DUB inhibitors (as indicated), crude cell extracts were prepared as described above with the addition of 35 μg GST-TUBE 1 or 2 (Lifesensors) to the lysate material (400 μg). After incubation on ice for 15 min, polyubiquitylated material was immunoprecipitated for 2 hr using glutathione affinity resin (Sigma-Aldrich) washed in TBS containing 0.1% NP40 and eluted using TBS containing 10 mM reduced glutathione (Sigma-Aldrich). Proteins were chloroform/methanol precipitated and kept at −80°C for further processing, either by in-solution digest (90%, see below) or resuspension in SDS-sample buffer (10%), separation by Bis-Tris 4%–12% SDS-PAGE, and analysis by anti-ubiquitin immunoblotting.

Sample Preparation and Analysis by Tandem Mass Spectrometry

Samples were subjected to in-solution trypsin digestion as described (Xu et al., 2008), and the analysis by tandem mass spectrometry (LC-MS/MS) is described in Supplemental Experimental Procedures.

Analysis of HDM2/p53/p21 and Claspin/pChk1Ser317

These experiments are described in Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and three tables and can be found with this article online at doi:10.1016/j.chembiol.2011.08.018.

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