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Generation and validation of intracellular ubiquitin variant inhibitors for USP7 and USP10

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**Abbreviations:** Ub, ubiquitin; UbV, ubiquitin variant; UPS, ubiquitin-proteasome system; DUB, deubiquitinase; USP, ubiquitin-specific protease; OTU, ovarian tumor; UCH, ubiquitin C-terminal hydrolase; ELISA, enzyme-linked immunosorbent assay; Ub-AMC, Ub with C-terminal derivatization of 7-amino-4-methylcoumarin; Ub-Rh110, Ub with C-terminal derivatization of Rhodamine 110 chloride; AP-MS, affinity purification mass spectrometry; WT, wild-type; IP, immunoprecipitation; EV, empty vector; tet, tetracycline; PI, propidium iodide; AV, Annexin V; DTT, dithiothreitol.
ABSTRACT

Post-translational modification of the p53 signaling pathway plays an important role in cell cycle progression and stress-induced apoptosis. Indeed, a large body of work has shown that dysregulation of p53 and its E3 ligase MDM2 by the ubiquitin-proteasome system (UPS) promotes carcinogenesis and malignant transformation. Thus, drug discovery efforts have focused on restoration of wild-type p53 activity or inactivation of oncogenic mutant p53 by targeted inhibition of UPS components, particularly key deubiquitinases (DUBs) of the ubiquitin-specific protease (USP) class. However, development of selective small molecule USP inhibitors has been challenging, partly due to the highly conserved structural features of the catalytic sites across the class. To tackle this problem, we devised a protein engineering strategy for rational design of inhibitors for DUBs and other UPS proteins. We employed a phage-displayed ubiquitin variant (UbV) library to develop inhibitors targeting the DUBs USP7 and USP10, which are involved in regulating levels of p53 and MDM2. We were able to identify UbVs that bound USP7 or USP10 with high affinity and inhibited deubiquitination activity. We solved the crystal structure of UbV.7.2 and rationalized the molecular basis for enhanced affinity and specificity for USP7. Finally, cell death was increased significantly by UbV.7.2 expression in a colon cancer cell line that was treated with the chemotherapy drug cisplatin, demonstrating the therapeutic potential of inhibiting USP7 by this approach.
INTRODUCTION

The tumor suppressor p53, known as the “guardian of the genome”, is a crucial transcription factor that induces cell cycle arrest and apoptosis in response to DNA damage and other stress signals [1, 2]. Therefore, in human cells p53 protein level and activity is tightly regulated to prevent carcinogenesis and halt malignant transformation. This is mainly controlled by ubiquitination, a highly coordinated post-translational modification pathway essential for many biological processes such as DNA damage checkpoint signaling, endocytic trafficking, and immune responses [3].

Ubiquitin (Ub) is a small (8.5 kDa), evolutionarily conserved protein that is at the center of this enzymatic cascade. Through the sequential actions of E1 activating enzymes, E2 conjugating enzymes, and E3 ligases, Ub chains or monomers are covalently attached to lysine residues or the N-termini of proteins, leading to protein degradation or signaling events [4-7]. Importantly, ubiquitination is reversed by deubiquitinases (DUBs), which catalyze removal of Ub from proteins [8].

While several E3 ligases contribute to p53 ubiquitination, including HUWE1 and WWP1, MDM2 is the dominant E3 ligase that ubiquitinates p53 to control its abundance and directly suppresses p53 transcriptional activity by blocking the N-terminal trans-activation domain of p53 [9-11]. On the other hand, the USP (Ub-specific protease) family DUB USP10 deubiquitinates and stabilizes p53 and reverses MDM2-induced p53 nuclear export and degradation [12]. USP10 also displays activity towards mutant p53 and promotes the oncogenic effects of mutant p53 in tumor cells [12]. Intriguingly, another DUB USP7 deubiquitinates both p53 and MDM2, the net effect of which is to stabilize MDM2 and consequently to inactivate p53 [13]. USP7 is therefore another critical factor in the p53-MDM2 signaling pathway that ensures the tightly controlled activity of MDM2 in order to maintain the proper balance of p53 protein level in cells [14]. Moreover, several other DUBs, including USP29, USP42 and the OTU (ovarian
tumor) protease OTUB1, also contribute to p53 deubiquitination in a context or cell line dependent manner [15].

Therapeutic targeting of the Ub-proteasome system (UPS) has been demonstrated by the success of proteasome inhibitors, such as Bortezomib, used for the treatment of multiple myeloma. However, the use of these drugs is limited to hypersensitive cancer cells with high protein synthesis rates (e.g., malignant plasma cells) and is associated with undesirable side effects [16, 17]. Thus, inhibitors of specific E3 ligases and DUBs in the UPS would greatly expand our pharmaceutical arsenal for cancer therapy, and could provide routes to better therapeutics with fewer side effects. For example, since many tumors that retain wild-type p53 show evidence of MDM2 overexpression [18, 19], targeting MDM2 in these cells is an attractive strategy for cancer therapy [2]. Nutlins represent the first group of small molecules that can interfere with the ability of MDM2 to mediate p53 ubiquitination and induce apoptosis and senescence in cancer cells [20]. Notably, Nutlins have a significant anti-tumor effect without obvious toxicity in mouse xenograft models [20], and Nutlin 3A has advanced to clinical trials for solid tumors and leukemia [21].

In the same vein, inhibition of USP7 in tumors with wild-type p53 is of interest for cancer therapy as it would result in elevation of p53 protein levels and restoration of its transcriptional activity, leading to either induction of cell death or potentiation of the effects of anti-cancer drugs [22, 23]. Indeed, small-molecule inhibitors that bind to the active site of USP7 have been reported, but these compounds exhibit low potency in the micromolar range [24]. After decades of drug development, only a handful of DUB inhibitors have been generated for the p53 signaling pathway and only MDM2 inhibitors have made it to clinical trials [25]. Collectively, these studies demonstrate the applicability of selective inhibitors of p53 ubiquitination as novel routes to improved cancer therapeutics, but they also highlight the paucity of a general strategy to develop potent and specific DUB inhibitors.
Recently, we have established a unified platform to target all Ub-interacting proteins [26]. We created a phage-displayed library of billions of Ub variants (UbVs) with mutations across the extended surface that interacts with DUBs and other UPS enzymes. Structural analysis of UbVs in complex with UPS components validated our strategy for the generation of specific inhibitors by optimization of intermolecular contacts through a combinatorial approach, which small molecules are unable to achieve. To date, we have generated UbV inhibitors or activators for ~50 UPS components spanning several protein families [26-29]. Here, we set out to establish a pipeline for discovery and characterization of intracellular inhibitors with therapeutic potential.

We focused on USP7 and USP10, which act as negative or positive regulators of p53 protein levels and activity, respectively [12, 24]. Inhibition of USP7 in tumors with wild-type p53 could result in elevation of p53 protein levels through destabilization of MDM2. This should restore p53 transcriptional activity, ultimately leading to induction of apoptosis or senescence. In contrast, in tumors that have oncogenic mutant p53, down-regulation of USP10 could be beneficial to prevent cancer progression by degradation of mutant p53. Indeed, p53 is mutated in more than 50% of human cancers, and it is evident that many mutant forms of p53 not only lose their tumor suppressing function and acquire dominant-negative activities, but also gain new oncogenic properties that are independent of wild-type p53 [30].

RESULTS

Potent and selective inhibitors for USP7 and USP10

We used a previously described UbV library [26] to screen for binders targeting full length human USP7 or USP10. From screening of 96 UbV clones following five rounds of binding selections (48 each from rounds 4 and 5), we were able to identify three UbVs that bound with high affinity to USP7 (UbV.7.1, 7.2 and 7.3) and one that bound to USP10 (UbV.10.1) (Fig. 1a). We assessed the binding specificity of UbVs against a panel of 11 DUBs including 7 USPs, 3 OTUs, and 1
UCH (ubiquitin C-terminal hydrolase) using phage enzyme-linked immunosorbent assays (ELISAs) and showed that each UbV bound detectably only to its cognate DUB (Fig. 1b). The UbVs bound to immobilized cognate DUBs with EC\textsubscript{50} values in the low nanomolar range, whereas wild-type Ub (Ub.wt) showed no binding to USP7 or USP10 in the assayed concentration range (up to 4 μM) (Fig. 1c and Table S1). Moreover, the UbVs potently inhibited the proteolytic activities of USP7 or USP10 towards the fluorogenic substrate Ub-AMC (7-amino-4-methylcoumarin) with IC\textsubscript{50} values in the sub-nanomolar range (Fig. 1d and Table S1), and they inhibited processing of K48- and K63-linked tetra-Ub substrates (Fig. 1e and 1f). It should be noted that USP10 is not a potent DUB for K48 linked chains (Fig. 1f), but rather prefers K63-linked or linear Ub chains [31].

**Potency and selectivity of UbV.7.2 far exceeds that of reported USP7 inhibitors**

Next, we assayed for the intracellular selectivity of USP7 UbVs by affinity purification mass spectrometry (AP-MS). We immunoprecipitated transiently expressed FLAG-tagged UbV.7.1, 7.2, or 7.3 in HEK293T cells and identified co-precipitated proteins by MS. Consistent with the binding and specificity data (Fig. 1b and 1c), all three UbVs co-immunoprecipitated endogenous USP7 but no other DUBs (Table S2). UbV.7.2 co-immunoprecipitated USP7 with the highest spectral counts among the three UbVs, together with many known USP7 interacting proteins such as PCNA and MCM7 (Table S2).

In our UbV library design, we only diversified solvent-exposed positions where substitutions would be unlikely to alter the protein fold. In contrast, the Corn group mutated the core β1/β2 loop of Ub [32, 33], which contributes to DUB binding to some extent but may also influence overall folding [34]. We noted that the best USP7 inhibitor generated by the Corn group (U7Ub25.2540) is non-specific. In a MS experiment performed in the same cell line as used by us, U7Ub25.2540 co-immunoprecipitated not only USP7, but also 10 other DUBs from several structural sub-families [34]. This prompted us to rigorously compare
UbV.7.2 and U7Ub25.2540 (see Fig. S1a for protein sequences) with in vitro enzymatic assays. In a Ub-AMC hydrolysis assay, UbV.7.2 inhibited USP7 activity more than 10-fold more potently than did U7Ub25.2540 (Fig. S1b and Table S1, IC$_{50}$ = 8.8 nM or 91 nM, respectively). We further confirmed this finding using K48- and K63-linked tetra-Ub substrates (Fig. S1c). Finally, we assessed the potency and selectivity of our three USP7 inhibitors and U7Ub25.2540 across a panel of 23 human DUBs and a deSUMOylase (SENP1) in deubiquitination assays using Ub-Rh (Rhodamine) 110 as the fluorogenic substrate (or SUMO2-Rh110 for SENP1), since the rhodamine fluorophore exhibits optical properties more appropriate for screening and profiling than does Ub-AMC [35]. UbV.7.2 inhibited USP7 more than 25-fold more potently than did U7Ub25.2540 (Table 1, IC$_{50}$ = 0.91 nM or 24 nM, respectively). Moreover, UbV.7.2 was far more selective than U7Ub25.2540, as it inhibited only two other DUBs significantly, whereas U7Ub25.2540 inhibited more than half of the DUBs that were screened (Table 1). Taken together, these results show that Ub 7.2 is the most potent and selective USP7 inhibitor developed to date.

**Structural analysis of UbV.7.2**

Previously we have solved several DUB-UbV complex structures and showed that UbVs tend to bind to the catalytic domain as mimics of Ub substrates [26]. Gel filtration showed that UbV.7.2 bound to the catalytic domain of USP7 (Fig. S2). Unfortunately, crystals of the USP7-UbV.7.2 complex did not diffract well enough to enable structure elucidation. However, we were able to solve the crystal structure of UbV.7.2 at 1.5 Å resolution in space group P 2$_1$ (Fig. 2a and Table 2). Each asymmetric unit contained two copies of UbV.7.2, which adopted the typical Ub fold. The RMSD (root-mean-square deviation) for all atoms of the first 73 amino acids of UbV7.2 when superimposed with Ub.wt (PDB: 1UBQ) was only 0.59 Å, showing that there was very little change in the conformation of the UbV compared to that of Ub.wt. Unexpectedly, residues 73–75 of UbV 7.2 formed a 3$_{10}$ helix structure, and the side chains of Asp$^{39}$ and Gln$^{40}$ formed
hydrogen bonds with the main chain amide group of Arg$^{77}$ and carbonyl group of Leu$^{73}$, respectively (Fig. S3). These interactions fixed the conformation of the C-terminal tail of UbV.7.2 in a different orientation compared to that of Ub.wt in apo-or USP7-conjugated forms (Fig. 2a).

We superposed the structure of UbV.7.2 with the complex structure of the USP7 catalytic domain covalently conjugated with Ub.wt (PDB: 5JTJ) [36] (Fig. 2b). The UbV.7.2 residues that were substituted relative to Ub.wt were mainly located in three regions: a hydrophobic patch comprised of Pro$^{2}$, Phe$^{63}$ and Ala$^{64}$, the shortest strand of the β-sheet on the side of the molecule that includes Lys$^{49}$ and Thr$^{46}$, and the C-terminal tail. A detailed inspection of the local interactions reveals potential enhanced interactions between UbV.7.2 and USP7.

The substitutions Gln$^{2}$Pro, Lys$^{63}$Phe and Glu$^{64}$Ala created a hydrophobic patch in an area that was hydrophilic in Ub.wt and interacted with the finger subdomain of USP7 (Fig. 2c). Removal of the side chain of Gln$^{2}$ may reduce the steric clash with the side chain of Asp$^{380}$ of USP7 (USP7 residues are denoted by an asterisks throughout), whereas the aromatic side chain of Phe$^{63}$ may favor a potential π-π stacking interaction [37] with the side chain of Tyr$^{379*}$. The side chain of Phe$^{63}$ may also form a π-cation interaction [38] with the side chain of Arg$^{343*}$. The importance of these three substitutions was confirmed by back mutation analysis, which showed that conversion of the three residues back to the wild-type sequence in the background of UbV.7.2 resulted in a ~300-fold reduction in binding affinity (Fig. S4 and Table S1, UbV.7.2-BM1). In addition, the substitution Gln$^{49}$Lys could potentially create a salt bridge between the side chains of Lys$^{49}$ and Glu$^{298*}$ on an α-helix of USP7 (Fig. 2d). This substitution occurs in all three USP7-binding UbVs (Fig. 1a), suggesting its importance for enhanced affinity, and indeed, back mutation to the wild-type sequence reduced affinity ~30-fold (Fig. S4 and Table S1, UbV.7.2-BM2).

Finally, the C-terminal region of UbV.7.2 contains substitutions with larger side chains (Ala$^{76}$, Arg$^{77}$ and Pro$^{78}$) than the C-terminal glycines found in Ub.wt, and thus, it is unlikely to fit in the narrow groove that leads to the catalytic triad of
USP7. Instead, formation of the $3_10$ helix and the consequent diverted orientation of the C-terminal region may enable the side chains of Arg$^{72}$ and Arg$^{74}$ to form salt bridges with the side chains of Asp$^{295\ast}$ and Glu$^{298\ast}$ on the switch helix of USP7 (Fig. S5). The side chain of Arg$^{74}$ may also form a hydrogen bond with the main chain of the C-terminal activation loop of USP7, and the side chain of Leu$^{73}$ may form hydrophobic interactions with Phe$^{409\ast}$ and Tyr$^{411\ast}$ on the blocking loop 1 of USP7. Back mutation of the C-terminal tail of UbV.7.2 to the wild-type sequence reduced binding affinity by $\sim$11-fold (Fig. S4 and Table S1, UbV.7.2-BM3). Moreover, simultaneous back mutation of residues in the three regions to the wild-type sequence reduced affinity even further (Fig. S4 and Table S1, UbV.7.2-BM4 and -BM5). In summary, a comparison of the crystal structure of UbV.7.2 with the USP7-Ub.wt complex, together with back mutation analysis of UbV.7.2, suggests that enhanced affinity for USP7 is due to new interactions mediated by a hydrophobic patch formed by Pro$^2$, Phe$^{63}$ and Ala$^{64}$, augmented by new interactions mediated by Lys$^{49}$ and the divergent C-terminal region.

UbV.7.2 induces MDM2 ubiquitination and stabilizes p53

To explore the effects of UbVs on USP7 and USP10 activity in cells, we first performed immunoprecipitation (IP) experiments in HEK293T cells harboring an empty vector (EV) or a vector expressing FLAG-tagged Ub.wt or UbV (7.1, 7.2, 7.3 or 10.1), all of which were un-conjugatable due to deletions or substitutions in the C-terminal di-glycine motif. UbV.10.1 co-precipitated endogenous USP10 but not USP7, while UbV.7.1, 7.2 and 7.3 co-precipitated endogenous USP7 but not USP10, albeit to different levels consistent with MS results (Fig. 3a). We also conducted cell growth assays and confirmed that UbV expression is not toxic to cells, consistent with the specific inhibition of cognate DUBs (Fig. S6). For subsequent studies, we focused on UbV.7.2, as it exhibited the best affinity and specificity in vitro (Table 1) and interacted strongly with endogenous USP7 (Fig. 3a).
We investigated the capacity of UbV.7.2 to inhibit USP7 catalytic activity in a cellular context. In HCT116 colon carcinoma cells with wild-type p53 (p53.wt), we transduced EV or a tetracycline (tet)-inducible lentiviral vector expressing UbV.7.2 (Fig. 3b). To ensure an accurate measurement of endogenous protein levels, we added cycloheximide (CHX) to block protein synthesis before assessment of protein turnover by collecting cell extracts at indicated time points (Fig. 3b). The expression of UbV.7.2 resulted in reduced MDM2 protein levels when compared with EV control. Consistently, p53.wt became more stable in these cells (Fig. 3b). It is quite remarkable to observe the dramatic reduction of MDM2 protein abundance, because MDM2 transcription can be activated by p53 (therefore p53 stabilization can cause higher MDM2 protein levels) [39]. To confirm whether the rapid MDM2 protein turnover was due to enhanced MDM2 ubiquitination, we immunoprecipitated MDM2 from HEK293T cells expressing UbV.7.2 and probed for ubiquitinated MDM2 by western blotting (Ub was expressed as HA-tagged). Indeed, UbV.7.2 expression significantly increased the amount of ubiquitinated MDM2 compared with cells harboring EV or a vector expressing Ub.wt, confirming the inhibition of USP7 activity (Fig. 3c).

**UbV.10.1 alters cellular localization and reduces stability of p53**

USP10 deubiquitinates p53.wt and mutant p53 and reverses MDM2-induced p53 nuclear export and degradation [12]. Therefore, an inhibitor of USP10 may render p53 unstable and shuttle p53 from nucleus to cytoplasm. In HCT116 cells, following 24 hours of tetracycline treatment to induce expression of UbVs, we confirmed that UbV.10.1 (but not a control UbV.2.5) co-immunoprecipitated USP10 (Fig. S7a) and also p53.wt (Fig. S7b). Next, we tested whether UbV.10.1 can modulate p53.wt function by binding to the USP10-p53.wt complex. As expected, expression of UbV.10.1 (but not a control UbV.2.5) initiated p53.wt nuclear export to the cytoplasm (Fig. 3d, at 24 hour time point). Consistently, this was accompanied by p53.wt degradation at later time point (Fig. 3e), presumably through the action of MDM2 [11, 40]. These results are consistent with the
USP10 knockdown phenotype using RNA interference [12], and confirmed that UbV.10.1 acts as an inhibitor of endogenous USP10 in cells.

Synergistic killing of cancer cells by UbV.7.2 and cisplatin

In response to chemotherapy, cancer cells die mainly by cell cycle arrest and apoptosis induced by p53 [1, 39]. However, partial cell killing often occurs and limits drug efficacy. Recently, it was found that cells must reach a threshold level of p53 to execute apoptosis, and cell-to-cell variations in p53 protein levels cause resistance to chemotherapy [41]. Importantly, it was also shown that cell death could be enhanced by increasing the rate of p53 protein accumulation in cells [41]. Because UbV.7.2 stabilizes p53, we hypothesized that expression of UbV.7.2 may increase cancer cell death potentiated by the chemotherapy drug cisplatin.

Following expression of UbV.7.2 induced by tetracycline for 24 hours, we treated HCT116 cells with low concentrations of cisplatin for 3 days and stained with Annexin V and propidium iodide (PI) to estimate cell death by flow cytometry. The expression of UbV.7.2 alone did not increase the number of dead cells when compared to EV or expression of Ub.wt, suggesting that UbV.7.2 is not toxic to cells (Fig. 4). In contrast, cells treated with cisplatin exhibited a higher number of dead cells relative to untreated cells, consistent with previous reports [41] (Fig. 4). Importantly, expression of UbV.7.2 further increased cell death in combination with cisplatin, including an increase in late apoptotic as well as necrotic cells (Fig. 4). Together, these data show that inhibition of USP7 by UbV.7.2 induces MDM2 depletion and p53 stabilization, which in turn increases sensitivity to cisplatin treatment and consequently leads to greater cell death.

DISCUSSION

Effective cancer therapies rely on our ability to interfere with cellular processes that are dysregulated in cancer cells. With more than 1200 human protein
components, the ubiquitin proteasome system (UPS) exerts profound and widespread effects on hundreds of signaling pathways within the proteome landscape and hence coordinates the majority of cellular processes. Indeed, numerous studies have shown that defects in the UPS promote many human diseases including immune disorders, neurodegeneration, and cancer [4, 42, 43]. The UPS thus represents a rich resource for novel therapeutic targets to enable new drug discovery programs. However, the clinical success of proteasome inhibitors notwithstanding, drug development in the UPS has lagged far behind efforts targeting phosphorylation and other post-translational modifications [21]. Thus, there is an urgent need for innovative approaches that can be used to interrogate specific components of the UPS to guide target identification, validation, and drug discovery. To fill this gap, we have devised UbV engineering as a general approach to developing inhibitors of virtually any UPS component [26-29, 43-45].

Here, we leveraged the UbV platform as an efficient pipeline for discovery and characterization of intracellular inhibitors of two USPs of high interest in oncology, which have proven resistant to conventional drug development efforts. Specifically, we targeted USP7 and USP10, critical modulators of the p53/MDM2 signaling pathway. Since the identification of the clinical candidate MDM2 [20] more than a decade ago, the pathway has resisted further therapeutic targeting, and there are only reports of a few USP7 inhibitors with low affinity and selectivity [46]. Through a pipeline encompassing phage display, structural analysis and cell-based assays, we demonstrated that extremely potent and specific UbV inhibitors for USP7 and USP10 could be developed rapidly. These UbVs functioned as inhibitors to target the p53/MDM2 pathway in cancer cells, and our best USP7 inhibitor sensitized cancer cells to low dose treatment with the chemotherapy drug cisplatin.

UbVs enable direct inhibition of catalytic activity, which cannot be achieved by genetic techniques such as RNAi or CRISPR that decrease mRNA and protein levels. Notably, different levels of protein down-regulation can lead to contrary observations. For example, moderate down-regulation of USP7 leads to p53
destabilization and thus favors cell proliferation [47] but complete loss of USP7 destabilizes MDM2, hence stabilizing p53 and inhibiting tumor growth [48]. Intriguingly, depletion of the DUB BAP1 can have severe unwanted pleiotropic effects, including destabilization of stoichiometric partners, as opposed to a direct inhibition of catalytic activity alone [49]. Thus, the UbV technology enables rapid and facile production of intracellular inhibitors that can empower target validation and drug discovery in the UPS. In conclusion, our study provided the most potent and selective intracellular inhibitors of USP7 and USP10 reported thus far, and the results demonstrate the power of applying UbV technology to rapidly target UPS components that have proven recalcitrant to conventional drug development methods.

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Materials and Methods

Ubiquitin variant selections

The phage-displayed UbV library used in this study was re-amplified from Library 2 [26]. Protein immobilization and following phage selections were done as previously described [28].

ELISAs to evaluate binding and specificity

Proteins were immobilized on 384-well MaxiSorp plates (Thermo Scientific 12665347) by adding 30 µL of 1 µM protein solution and incubating overnight at 4°C. Phage and protein ELISAs with immobilized proteins were performed as described [26, 28]. Binding of phage or FLAG-tagged UbVs was detected using anti-M13-HRP (GE Healthcare 27942101) or anti-FLAG-HRP antibody (Sigma-Aldrich A8592), respectively. To measure the half maximal effective concentration (EC$_{50}$) of UbVs binding to immobilized DUBs, the concentration of UbVs in solution was varied from 0 to 4 µM by two-fold serial dilutions, and EC$_{50}$ values were calculated using the GraphPad Prism software with the built-in equation formula (non-linear regression curve).

Proteolytic assays

Proteolysis inhibition assays using Ub-AMC (both Boston Biochem) as substrate were performed as described [26, 45]. Experiments were performed in assay buffer (50 mM HEPES pH 7.5, 0.01% Tween 20, 1 mM dithiothreitol (DTT)) containing 1 µM Ub-AMC substrate, 10 nM DUB, and serial dilutions of UbV protein. DUB and UbV protein were mixed in assay buffer as indicated and incubated at room temperature for 2 min prior to the addition of Ub-AMC. Serial dilutions were performed in 96-well plates (Thermo Fisher Scientific) and subsequently transferred to 384-well black plates (Thermo Scientific) for making measurements. Proteolytic activity was measured by monitoring the increase of AMC fluorescence emission at 460 nm (excitation at 360 nm) for 30 min using a BioTek Synergy2 plate reader (BioTek Instruments, Winooski, VT). IC$_{50}$ values were calculated using the GraphPad Prism software with the built-in equation
formula (non-linear regression curve). To directly compare 7.2 and U7Ub25.2540, Ub-AMC assay was performed according to protocols described in Zhang et al. [33].

Inhibition assays using biotinylated K48- or K63-linked tetra-Ub chains (Boston Biochem) as substrates were performed in assay buffer (50 mM HEPES pH 7.5, 100 mM NaCl, 1 mM DTT) containing 1 μM substrate, 1 μM vDUB and 10 μM UbV. After incubation at 37°C for the indicated times, reactions were stopped by the addition of 10 mM EDTA and SDS-PAGE sample buffer and resolved using 4-20% gradient gels (Bio-Rad). The cleavage of tetra-Ub chains was evaluated by western blots probed with ExtrAvidin®-Peroxidase (Sigma).

**Protein expression and purification**

Plasmid pET53-UbV.7.1, 7.2, 7.3 or 10.1 was transformed into CaCl2-competent *Escherichia coli* BL21 (DE3) Gold cells (Agilent) to enable T7 polymerase-driven expression of N-terminally His6-tagged UbVs. Cells were grown at 37°C in the presence of 150 μg/mL ampicillin to an optical density (OD600) of 0.6 and induced at 16°C by addition of 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG). After overnight incubation, cells were resuspended in lysis buffer (50 mM Tris pH 8.0, 500 mM NaCl) and lysed with a French press. The cell lysate was clarified by centrifugation at 17,200 x g at 4°C for 30 min, incubated with 2 mL Ni-NTA Superflow resin (Qiagen) at 4°C for 30 min, and poured into a gravity column. The column was washed with 50 mL of lysis buffer, followed by 50 mL of lysis buffer containing 50 mM imidazole, and protein was eluted in lysis buffer containing 250 mM imidazole. UbVs were further purified using a Superdex 75 size exclusion column (GE Healthcare), eluting in 20 mM Tris pH 8.5, 150 mM NaCl, 2 mM DTT.

For crystallization experiments described below, DNA fragments encoding UbV.7.2 or USP7 catalytic domain (CD) were subcloned into pET28-MHL (EF456735, for TEV protease cleavable N-terminal His6-tag) or pET28a-LIC (EF442785, for thrombin cleavable N-terminal His6-tag) vectors, respectively.
Proteins were produced in *E. coli* BL21(DE3) harboring a pRARE2 plasmid using Terrific Broth cultured in LEX Bioreactors (Harbinger Biotech). Protein production was induced with 0.5 mM IPTG overnight at 18°C. Proteins were first purified using nickel-nitrilotriacetic acid (Ni-NTA) agarose resin (Qiagen), and the His6-tag of UbV.7.2 was removed by TEV protease (1:30 w/w). Uncleaved protein and TEV protease were removed by another pass through the Ni-NTA resin. The proteins were further purified using anion-exchange chromatography (Source 30Q, GE Healthcare) and size-exclusion chromatography. The purified proteins were concentrated to a final concentration of 20–30 mg/mL in 25 mM HEPES pH 8.0, 150 mM NaCl, 1 mM DTT and stored at -80°C. Protein concentrations were determined by measuring UV absorbance at 280 nm using NanoDrop 2000 (Thermo Scientific).

**Deubiquitinase panel screening**

*Materials*

Deubiquitinases (DUBs) were generated and purified in-house at GSK or obtained from LifeSensors (Malvern, PA) or Ubiquigent (Dundee, Scotland, UK). See Table S3 for details. Ubiquitin-Rhodamine110 (“Ub-R110”) substrate was obtained from Boston Biochem, Inc. (Cambridge, MA). SUMO2-Rhodamine110 substrate was obtained from LifeSensors and used only for SENP1 reactions. HEPES buffer solution was obtained from Teknova. EDTA solution was obtained from Promega. TCEP HCl was obtained from Pierce/Life Technologies. DTT was obtained from Fisher BioReagents. TRIS buffer solution (Trizma®-HCl, pH 7.5), BCA, CHAPS, and NaCl were obtained from Sigma-Aldrich. Assay plates were obtained from Greiner Bio-One.

*DUB enzyme panel inhibition assay*

DUB reactions were run in assay buffer A containing 50 mM HEPES, pH 7.5, 0.5 mM EDTA, 0.01% BSA, 0.8 mM CHAPS, and 0.5 mM TCEP. UCHL3 reactions were run in assay buffer B containing 50 mM TRIS, pH 7.5, 6 mM EDTA, 0.0067% BSA, 0.8 mM CHAPS, and 4 mM DTT. SENP1 reactions were run in
assay buffer C containing 50 mM HEPES, pH 7.5, 100 mM NaCl, 0.01% BSA, 1 mM CHAPS, and 0.5 mM TCEP.

Enzyme concentration and substrate concentration were optimized by co-titration. Enzyme titration was used to determine optimal activity and reaction linearity. Ub-R110 (or SUMO2-R110 for SENP1) substrate titration was used to determine substrate Km value. Substrate concentration for assay was fixed at approximately 1xKm, unless Km > 1 uM, where substrate concentration was then fixed at 1 uM for practical considerations, such as cost, background fluorescence, and addition of DMSO to assay (substrate solubilized in 100% DMSO at 112 uM concentration; 0.9% final with 1 uM concentration in assay). See Table S4 for details.

Enzyme and substrate solutions for 25 different enzyme reactions were prepared in bulk at 2.4x assay concentrations in the appropriate assay buffer. Reagents were aliquoted to storage plates (Greiner #781280) in volumes suitable to run one assay testing up to six test compounds. Plates were sealed with foil seals, frozen on dry ice, and stored at -80°C. After -80°C storage for >24 hours, a test plate was thawed at room temperature, and enzyme activity was tested and compared to activity of freshly prepared enzyme and substrate. Results showed < 2x loss of activity and comparable reaction kinetics for all enzymes (data not shown).

Immediately prior to assay, a frozen reagent plate was taken from -80°C storage and thawed at room temperature. UbV 11-point dilutions were prepared in 1x Assay Buffer A at 6x concentrations, with a top concentration of 60 uM (10 uM final), 3x dilution scheme, and a resulting lowest concentration of 1.0 nM (0.17 nM final). UbV dilutions were added to 384-well black assay plates (Greiner #784076) in 1.6 ul volume. 4 ul 2.4x enzyme mix was added to 1.6 ul Ubv and pre-incubated for 30 minutes at room temperature. After pre-incubation of enzyme and UbV, 4 ul 2.4x substrate mix was added to initiate reaction. Assay plate was briefly spun in centrifuge (30 seconds at 1000 RPM) to settle and mix reagents. Plate was then read in Envision plate reader (Excitation 485 nm,
Emission 535 nm), using kinetic read at 1 minute intervals for 40 minutes. Positive and negative control reactions (Buffer + Enzyme + Substrate, and Buffer + Substrate only, respectively) were included for each enzyme. Duplicate (N=2) analyses were conducted using frozen reagent plates prepared at the same time, but using freshly prepared titrations of Ub variant.

For analysis of each enzyme, positive control reactions were analyzed to determine duration of linear reaction, and rates were determined for this time period for each reaction. Rates of negative control reactions were also determined for background-subtraction, although no significant rate was observed (varying substrate concentrations change raw background signal, but no turn-over was observed over time). Background-subtracted rates were converted to percent of positive control (control set to 100%) and fit to Equation 1 to determine IC$_{50}$ values. Fitting constraints used are shown below.

**Equation 1.** $Y=Y_{\text{min}} + \frac{(Y_{\text{max}}-Y_{\text{min}})}{1+10^{((\log IC_{50}-X)\times \text{Hill Slope})}}$

Where $X$ is the logarithm of concentration and $Y$ is the response

**IC$_{50}$ fitting constraints**

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<th>PARAMETER</th>
<th>CONSTRAINT</th>
</tr>
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</tr>
<tr>
<td>$Y_{\text{max}}$</td>
<td>&lt; 105</td>
</tr>
<tr>
<td>Log [IC$_{50}$]</td>
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<tr>
<td>Hill Slope</td>
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</table>

**Crystallization, data collection, structure determination, refinement and analysis**

Rod-shaped crystals of UbV.7.2 were grown at 20°C using the sitting drop vaporization method by mixing 0.5 μL each of the protein and the well solutions
containing 0.1 M Tris-HCl pH 8.0, 1.2 M sodium citrate. Harvested crystal was cryo-protected by immersion in a drop containing the well solution plus 10% ethylene glycol and flash-frozen in liquid nitrogen. X-ray diffraction data were collected at beamline 19-ID of the Advanced Photon Source, Argonne National Laboratory. The datasets were processed with the HKL-3000 suite [50]. The structure was solved by molecular replacement using PHASER [51] with PDB entry 1UBQ as the search template. COOT [52] was used for model building and visualization, and REFMAC [53] was used for restrained refinement. The final models were validated by MOLPROBITY [54].

Cell culture

HEK293T cells were cultured in Dulbecco’s modified Eagles medium (DMEM) and HCT116 cells were cultured in McCoy’s 5A medium. Both media were supplemented with 10% fetal bovine serum (FBS), 100 U/ml of penicillin and 100 μg/ml of streptomycin. All cell lines were maintained at 37°C in an incubator with a humidified atmosphere of 5% CO₂ in air.

Transient cell transfection, immunoprecipitation and immunoblotting

For transient expression, a DNA fragment encoding a UbV or Ub lacking C-terminal di-Gly were cloned into pDEST-pcDNA3.1/nFLAG vector and a DNA fragment encoding Ub.wt was cloned into pDEST-pcDNA3.1/nHA vector (HA-wt-Ub). These vectors were obtained from SPARC Biocentre (Hospital for Sick Children, Toronto, Canada). Cloning was performed using the Gateway system (Invitrogen), and all clones were verified by DNA sequencing.

HEK293T cells were grown to 60-70% confluency on 6-well plates and transfected with 1-2 ug plasmids for expression of UbV or control plasmids (EV and Ub), together with 0.2 ug plasmid for expression of HA-wt-Ub for the ubiquitination assay, using the XtremeGENE transfection reagent (Roche 06365809001) according to manufacturer's instructions.

Cells were harvested 2 days post-transfection, washed in PBS and resuspended in lysis buffer (50mM Tris pH 8.0, 150 mM NaCl, 1% NP40, 4.5 mM NaVO₄, 4.5
mM Na Pyrophosphate, 22.5 mM NaF, Halt Protease Inhibitor Cocktail (Thermo scientific 78430)). Lysates were centrifuged at 4°C for 10 min at 12,000 rpm. The supernatant was transferred to a new tube and protein concentration was determined by Bradford assay and standardized to 1 µg/µl.

For immunoprecipitation assay, the lysates were incubated with anti-FLAG M2 agarose resin (A2220 Sigma) overnight at 4°C. Alternatively, the lysates were incubated with specific antibody overnight at 4°C followed by 2 hours incubation with protein A/G agarose beads (#20423, Thermo Scientific). The agarose pellets were washed 3 times in lysis buffer. LDS sample buffer (NP0007, Life Technologies) with 50 mM DTT was added to whole cell lysates and immunoprecipitated samples. Samples were heated at 70°C for 10 min and loaded onto gels for western blotting. The following antibodies were used: anti-USP7 (11984S, Cell Signalling), anti-USP10 (5553S, Cell Signalling), anti-beta-ACTIN (MA5-15739, Thermo scientific), anti-FLAG (ab49763, Abcam), anti-MDM2 (sc-813, Santa Cruz Technology), anti-P53 (13-4000, Invitrogen), anti-HA (sc-7392, Santa Cruz Technology).

**Stable cell lines and cell death analysis**

For stable expression, a DNA fragment encoding UbV.7.2 or Ub was cloned into pLJM17 lentiviral vector containing a tetracycline-inducible promoter [55]. Colon carcinoma HCT116 cells were transduced with lentivirus for expression of UbV.7.2, Ub or EV. Cells were treated with 1 ug/ml of doxycycline, a tetracycline derivative, for 24 hours to induce gene expression. To analyze protein levels and turnover, induced cells were treated with 100 ug/ml of protein synthesis inhibitor cycloheximde (CHX) and collected at indicated time points. Cell pellets were processed for western blotting as described above. To analyze cell death, induced cells were treated with 14 uM cisplatin for 3 days. Treated cells were trypsinized, counted, washed with PBS and stained with Annexin V (AV) and Propidium Iodide (PI) (V13242, Thermo Scientific) according to manufacturer’s instructions. Stained cells were analyzed by UV excitation at 355 nm on a BD LSRFortessa X-20 cell analyzer and detected using a 450/50 nm bandpass filter.
The data acquired was analyzed by FlowJo10 software (Treestar Inc.). Percentages of total dead cells, necrosis (PI only), late apoptosis (Annexin V+PI) or early apoptosis (Annexin V only) were normalized by untreated cells. The graph combines data from 3 biological replicates with 3 technical replicates each.

**Confocal microscopy**

HCT116 stable cells were seeded on poly-L-lysine treated glass coverslips at concentrations of $5 \times 10^4$ cells per well in 24-well plates. After 16 hr, cells were treated with tet (0.5 ug/mL) or vehicle for 24hr. For recovery experiments, dox-induced cells were washed twice with 1mL PBS and were then cultured in regular medium for 24 hr. Cells were washed twice with 1 mL PBS and fixed with cold 95% methanol for 20 min on ice before blocking with 5% BSA for 1 hr. After 3 washes with PBS, cells were permeabilized with 0.1% Triton X-100 for 10 min and incubated for 1 hr at room temperature with mouse monoclonal anti-p53 antibody (1:200, 13-4100, Invitrogen). After 3 washes with PBS, cells were incubated with goat anti-mouse Alexa 568 Fluor-conjugated secondary antibody (1:1000, A11004, Life Technologies) and briefly stained with DAPI. Coverslips were mounted with SlowFade Diamond antifade mountant (Life Technologies). Images were acquired using a Quorum WAveFX-X1 spinning disc confocal system at 60x magnification with an Olympus S-Apo 60x/1.35 oil objective (Quorum Technologies Inc., Guelph, Canada).

**Affinity purification-mass spectrometry (AP-MS)**

Samples preparation, purification, mass spectrometry analysis and data processing were performed as described [56].

**ACCESSION NUMBERS:** Coordinates and structure factors for UbV.7.2 have been deposited in the Protein Data Bank with accession number 5VBT.
Figure Legends

Figure 1. UbVs inhibit USP enzyme activity in vitro. (a) Sequences of UbVs that bind USP7 or USP10. Only regions subjected to diversification relative to Ub.wt in the phage-displayed library are shown and dashes indicate conservation of the Ub.wt sequence. (b) The binding specificities of phage-displayed UbVs (y-axis) are shown across a group of 11 DUBs (x-axis), as assessed by phage ELISA. Sub-saturating concentrations of UbV-phage were added to immobilized proteins as indicated. Bound phages were detected by the addition of anti-M13-HRP and colorimetric development of TMB peroxidase substrate. The mean value of absorbance at 450 nm is shaded in a white-purple gradient. (c) Binding curves of UbVs to the cognate proteins measured by ELISA. The half maximal effective binding concentration (EC$_{50}$) of each UbV to the indicated DUB was determined by established methods [28] and listed in Table S1. USP7 or USP10 (1 μM) were immobilized in microtiter plates. Two-fold serial dilutions of FLAG-tagged UbV or Ub.wt (starting at 4 μM) were added and incubated for 20 min at room temperature. Wells were washed and bound UbV was detected by anti-FLAG-HRP conjugate antibody and colorimetric development of TMB peroxidase substrate. The absorbance at 450 nm (y-axis) was plotted against UbV concentration (x-axis). Data were presented as the mean ± SD (N = 3). (d) Inhibition of USP7 or USP10 proteolytic activity by the cognate UbVs shown as dose-response curves using Ub-AMC as the substrate. The IC$_{50}$ value was determined as the concentration of UbV that inhibited 50% of proteolytic activity (Table S1). (e and f) Effects of UbV inhibitors on DUB activity of USP7 (e) or USP10 (f) against K48- (top) or K63-linked (bottom) tetra-Ub (Ub4) substrates. Purified DUB protein was incubated with the indicated UbV or Ub.wt (negative control) and biotinylated tetra-Ub at 37°C for a time course of 30 minutes. Western blots were probed with ExtrAvidin-HRP (EA-HRP) to detect biotin-Ub. Inhibition of proteolysis was indicated by a delay of appearance of the digestion products tri-Ub (Ub3), di-Ub (Ub2) and mono-Ub (Ub1).

Figure 2. Structural analysis of UbV.7.2. (a) Crystal structure of UbV.7.2 (purple) superposed with Ub.wt from the structure of the USP7~Ub.wt covalent
adduct (orange). Residues in UbV.7.2 that were substituted relative to Ub.wt are shown as stick-and-balls and labeled. The size of the tube corresponds to the B factor of the main chain. (b) Modeling of the UbV.7.2 structure docked with the USP7~Ub.wt complex structure. The two blocking loops of USP7 (b-loop 1 and 2) are colored red, a switch loop is colored lime, the rest of the molecule is colored yellow, and the triad is shown as sticks. Potential key interactions between UbV.7.2 and USP7 within the black and red boxed regions are shown in (c) and (d), respectively. (c) Modeling of the interactions between UbV.7.2 and the finger domain of USP7. The side chains of UbV.7.2 are shown as stick-and-balls, and those of USP7 are shown as sticks and labeled with asterisks. (d) Modeling of the interactions between UbV.7.2 and an α-helix of USP7. The amine group of the Lys^{49} side chain is 2.5 Å from the carboxylic acid group of Glu^{298} and could form a salt bridge.

**Figure 3. UbVs inhibit USP activity in cells.** (a) HEK293T cells were transfected with Flag-tagged UbV or control plasmids. Protein complexes were immunoprecipitated (IP) from cell lysates using anti-Flag resin and probed with antibodies against USP7 or USP10. UbV.7.1, 7.2 and 7.3 immunoprecipitated endogenous USP7, and UbV.10.1 immunoprecipitated endogenous USP10. Negative controls, empty vector (EV) and control Ub (lacking the C-terminal di-glycine) did not immunoprecipitate USP7 or USP10. (b) HCT116 cells with an EV negative control or stably expressing UbV.7.2 under the control of a tetracycline (tet)-inducible promoter were induced for 24 hours. Cells were treated with cycloheximide (CHX) for the indicated time points and cell lysates were probed with antibodies against endogenous MDM2, p53, ACTIN (loading control) or FLAG (UbV). Expression of UbV.7.2 caused a reduction in MDM2 and stabilization of p53 protein levels. (c) HEK293T cells were transfected with vectors expressing Flag-tagged UbV.7.2 (or Ub or EV as negative controls) and HA-tagged Ub (HA-Ub). Cell lysates were subjected to MDM2 IP and probed with anti-HA antibody to detect ubiquitinated MDM2. (d) Expression of UbV.10.1 induces translocation of p53 from the nucleus to the cytoplasm. HCT116 cells stably expressing FLAG-tagged UbV.10.1 or UbV.2.5 under control of the tet-
inducible promoter were induced for 24 hours followed by recovery for 24 hours, then fixed and stained as indicated. Scale bar represents 10 µm. (e) Expression of UbV.10.1 reduces p53 levels. HCT116 cells stably expressing FLAG-tagged UbV.10.1 under the tet-inducible promoter were induced for 48 hours followed by recovery for 24 hours. Whole cell lysates were prepared at indicated time points, separated by SDS-PAGE and analyzed by western blotting.

**Figure 4. UbV.7.2 induces synergistic cell killing with cisplatin.** HCT116 cells stably expressing UbV.7.2, EV or Ub under the tet-inducible promoter were induced for 24 hours where indicated (tet). Cells were treated with only cisplatin (cis) for 3 days or with tet for 24 hours followed by cisplatin for 3 days (tet+cis). Annexin V (AV) and propidium iodide (PI) were added to the cells and samples were analyzed by flow cytometry. Percentages of total dead cells, necrotic cells (PI only), late apoptotic cells (AV + PI) or early apoptotic cells (AV only) were normalized by untreated cells. Expression of UbV.7.2 or controls (tet) did not increase cell death while treatment with cisplatin (cis) increased cell death in all conditions. Expression of UbV.7.2 enhanced cisplatin-induced cell death by both apoptosis and necrosis (tet+cis).
REFERENCES

Figure 1

(a) Table showing regions of interest with amino acid sequences.

(b) Heatmap indicating binding levels across different samples.

(c) Graph showing binding affinity over concentration.

(d) Graph showing inhibition of UbV over concentration.

(e) Gel images showing reactions at different times.

(f) Additional gel images showing different samples and exposure times.
Figure 4

Total Dead Cells

Necrosis

Late apoptosis

Early apoptosis

Fold change relative to untreated cells
Graphical abstract
Table 1. IC50 values of USP7 Ubvs for inhibition of USP7 activity (Ub-Rhodamine 110 as deubiquitination substrate).

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<th>Human DUBs</th>
<th>IC50 (nM, mean)</th>
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<tbody>
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<td>USP7</td>
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1′U7Ub25.2540  
2′Catalytic domain only  
3′Full length minus transmembrane domain (1-1290)  
4′Bold Italic: IC50<10 mM  
5′Bold: 500 nM>IC50>10 mM
Table 2. X-ray data collection and refinement statistics

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<tr>
<td>Redundancy</td>
<td>5.3 (4.5)</td>
</tr>
</tbody>
</table>

| Refinement | |
| Resolution (Å) | 50.0–1.50 |
| No. reflections | 21344 (1263) |
| R_work / R_free | 0.164/0.192 |
| No. atoms / average B-factors (Å²) | 1414/18.5 |
| Protein | 1277/17.6 |
| Water | 137/27.4 |
| R.m.s. deviations | |
| Bond lengths (Å) | 0.008 |
| Bond angles (°) | 1.428 |
| Ramachandran plot | |
| Favored regions (%) | 100.0 |
| Allowed regions (%) | 0.0 |
| Disallowed regions (%) | 0.0 |

* Values in parentheses are for highest-resolution bin.
Generation and validation of intracellular ubiquitin variant inhibitors for USP7 and USP10

Zhang et al.

HIGHLIGHTS

- Potent and selective inhibitors were developed for USP7 and USP10
- Crystal structure of UbV.7.2 sheds light on binding and inhibition mechanism
- UbV.7.2 destabilizes MDM2 and stabilizes p53
- UbV.10.1 affects p53 localization and induces p53 degradation
- UbV.7.2 enhances DNA damage-induced cancer cell death