Quantitative Chemical Proteomic Profiling of Ubiquitin Specific Proteases in Intact Cancer Cells

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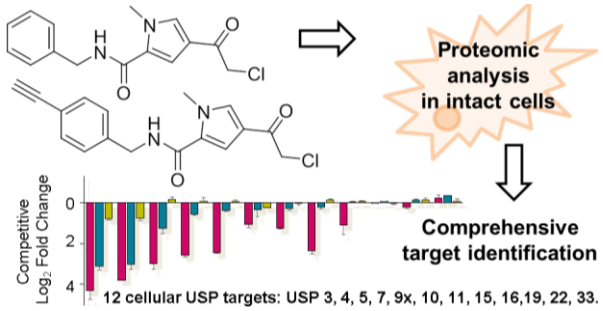

Abstract

Deubiquitinating enzymes play an important role in a plethora of therapeutically relevant processes, and are emerging as pioneering drug targets. Herein, we present a novel probe Ubiquitin Specific Protease (USP) inhibitor, alongside an alkyne-tagged activity-based probe analogue. Activity-based proteome profiling identified 12 USPs, including USP4, USP16, and USP33, as inhibitor targets using sub-micromolar probe concentrations. This represents the first intact cell activity-based profiling of deubiquitinating enzymes. Further analysis demonstrated functional inhibition of USP33 and identified a synergistic relationship in combination with ATR inhibition, consistent with USP4 inhibition.

Protein ubiquitination, the post-translational addition of the 76 amino acid protein ubiquitin (Ub), is a widespread protein post-translational modification (PTM) in eukaryotic cells.[1] Ub processing can result in varied linear and branched oligomeric chains, and regulation of this motif serves to modulate both protein half-life through the ubiquitin-proteasome system, and a multitude of important signalling pathways, for example during activation of the DNA damage response (DDR).[2] Deubiquitinating enzymes (DUBs) are responsible for removal of Ub marks, and are emerging as pioneering drug targets and potential biomarkers across many therapeutic areas, notably cancer.[3-5] The discovery and development of novel DUB inhibitors has benefitted from Ub-derived activity-based probes (ABPs) such as HA-Ub-VME, an HA (epitope)-tagged Ub carrying a C-terminal vinylmethyl ester warhead.[6-10] These ABPs form extensive interactions with their targets and covalently label the active site DUB cysteine, enabling DUB activity to be assayed in isolation or profiled in cell lysates.[11, 12] However, Ub protein-based probes are membrane-impermeable,[13, 14] and there is an unmet need for ABPs that can profile DUB activity and target engagement directly in the complexity of an intact cell.

Here we report, to our knowledge, the first cell-permeable small molecule ABP for the Ubiquitin Specific Protease (USP) family of DUBs (**2**). We demonstrate that this probe class efficiently labels a range of USPs in a native cancer cell environment at sub-micromolar concentrations, and is an effective tool to profile USP inhibitor target engagement through quantitative chemical proteomic competition experiments. In addition to comprehensive identification of targets in intact cancer cells, target confirmation and functional assays enabled validation of a novel irreversible USP inhibitor (**1**) that showed synergy with inhibition of ATR, a kinase mediating the DDR, consistent with activity against USP4 in osteosarcoma cells.

Following a high-throughput screening (HTS) campaign to identify DUB inhibitors, we synthesised 4-chloroacetylpyrrole derivative **1** (Figure 1A) as a follow-up to an initial HTS hit. Compound **1** is a sub-micromolar inhibitor of USP4 and low micromolar inhibitor of its phylogenetic relative USP11 (Supplementary Figure 1) in a biochemical IC_{50} assay. Consistently, low micromolar inhibition of USP4 and USP11 is observed in human osteosarcoma (U2OS) cells, as determined by HA-Ub-VME ABP labelling following cell lysis (Figure 1B). As a putative irreversible inhibitor we hypothesised that **1** offers a scaffold for profiling the activity of these USPs in cells, and an alkyne-tagged analogue **2** (Figure 1C) was synthesised to investigate the potential of this chemotype as a USP ABP. Formation of a covalent adduct was confirmed by electrospray mass spectrometry (MS) analysis of recombinant USP11 after incubation with **2** (Supplementary Figure 2). Compound **2** retains inhibitory potency towards USP4 and USP11, observed by a fluorescence polarisation recombinant DUB activity assay using an Ub-TAMRA substrate, whilst negligible inhibition was observed for control compound **3**, which lacks the chloromethylketone covalent warhead (Supplementary Table 1, Supplementary Figure 3).

Prior to proteomic profiling experiments, we first determined a suitable probe labelling concentration. Compound **2** was incubated at varying concentrations with U2OS cells for 1 hour prior to cell lysis, followed by copper catalysed azide–alkyne cycloaddition (CuAAC) ligation of probe-protein complexes to our previously reported trifunctional capture reagent azido–TAMRA–biotin AzTB (Supplementary Figure 4).[15] Visualisation by in-gel fluorescence revealed

that **2** labels a number of targets at sub-micromolar concentrations (Supplementary Figure 5). A concentration of 125 nM was selected for follow-up experiments to ensure sufficient labelling whilst maximising the range for competitive activity-based proteome profiling (ABPP). Loss of labelling in heat-treated lysate confirmed the requirement for native protein folding (Supplementary Figure 6).

Competition studies by in-gel fluorescence demonstrated that **1** caused a dose-dependent reduction in labelling intensity for specific protein bands at sub-micromolar concentrations (Figure 1D), whilst no competition was observed against **3** (Supplementary Figure 7). Proteome-wide in-cell target identification was performed using spike-in SILAC methodology,[16] as previously described.[17, 18] U2OS cells were treated for 1 hour with 125 nM of **2** after a 30 minute pre-incubation with a fixed concentration of **1** (0 nM, 125 nM, 625 nM, 5 μM, 10 μM) or 5 μM of **3**. Competition lysates were mixed in a 2:1 ratio with **2**-treated 'spike' prior to CuAAC ligation, and **2**-labelled proteins were enriched on NeutrAvidin-Agarose resin and trypsin digested.

Nanoscale liquid chromatography-MS/MS analysis showed that 125 nM **2** labels 12 USPs in intact cells (Figure 2A, Supplementary Dataset 1), a selection of which were validated through a fluorescence-intensity recombinant DUB activity assay, using an Ub-Rhodamine substrate (Table 1). By comparison, although HA-Ub-VME (9,913 Da) labels a broader range of DUBs in cell lysate, **1** (314 Da) demonstrates a much higher ligand efficiency than its Ub-based counterpart, and uniquely enables intact cell USP profiling. Despite its small size, USP labelling by **2** is considerably more specific than widely used cysteine-reactive small molecule probes; for example, alkyne-tagged iodoacetamide has been reported to competitively label 3 USPs at 10 μM in cancer cell lysates, whilst a recently reported caged-BK derivative labels 2 USPs at 200 μM in HeLa cells.[19, 20] Profiling the activity of **2** against an extended panel of recombinant DUBs identified additional USP targets (USP2, USP6, USP8, USP13, USP20, and USP30) with inherently low expression in U2OS cells[21] (Supplementary Table 2). Conversely **2** demonstrated low potency when profiled against alternative DUB families, suggesting that this probe has a preference for USPs.

Compound **2** displays a broad detection range in competitive ABPP, with significant competition observed from 1- to 80-fold excess **1**. Competition with 5 μM of **3** was negligible compared to analogous treatment with **1** (Supplementary Figure 8). As predicted, USP4 showed significant competition, whilst USP16 and USP33 were unexpectedly identified as the most potent USP targets of **1**. USP11 was also significantly competed, along with USP3, USP10, USP15, USP19, and USP22. USP5 and USP9x, though identified by **2**, were not significantly competed across this competition range, whilst USP7 showed only limited competition at 10 μM (data not shown). Variable expression of the aforementioned USPs has previously been implicated in cancer, including roles in p53 signalling,[4] metastatic grade,[22] and the hypoxic response.[23, 24] The competitive probe engagement observed by ABPP was validated for a selection of these USPs by Western blot (Figure 2B), and newly identified USP targets of **1** were further validated by HA-Ub-VME ABP labelling (Figure 2C, Supplementary Figure 9).

In total, on competition with 625 nM of **1**, 27 significantly competed hits were identified (Supplementary Table 3, Supplementary Figure 10). These hits revealed significant enrichment

for oxidation-reduction processes, cell redox homeostasis, and protein deubiquitination biological GO terms by STRING network analysis (Figure 2D). As well as the USP targets already described, redox process enzymes including 5 thioredoxins, related nucleoredoxin[25] and 2 glutathione transferase domains were competed, as might be expected for this electrophilic chemotype. PTGES2, which showed the greatest ratio fold change, contains the consensus region and the active site cysteine of the redox enzymes glutaredoxin and thioredoxin.[26] A full list of the 27 protein targets and associated GO terms is detailed in Supplementary Table 3.

We next examined functional inhibition of a novel USP target by **1** in U2OS cells. USP33 has been reported to deubiquitinate and consequently stabilise CP110, a key regulator of centrosome duplication and thus genomic stability.[27] U2OS cells were treated with DMSO or **1** (10 μM) for 24, 48 and 72 hours, and cell lysates analysed by western blot (Figure 3A). Compound **1**-treatment led to decreased CP110 protein levels, consistent with decreased stability following USP33 inhibition.

USPs which regulate genomic integrity show promise as drug targets in combination with known DNA damaging chemotherapeutics and through synthetic lethal approaches.[3] For example, USP4 promotes homologous recombination (HR) of DNA double strand breaks (DSBs),[28, 29] and USP16 has also been implicated in DSB repair[30]: both of which are prominent USP targets of **1** in U2OS cells by competitive ABPP. ATM and ATR are DNA damage checkpoint kinases that regulate the response to DSBs and DNA damage induced replication stress respectively, whose inhibition has been investigated as an approach for tumour cell sensitisation.[31] For example, ATR inhibition has been previously reported to induce synthetic lethality in ATM deficient cells.[32-34] Profiling the antiproliferative effects of **1** in HeLa cells in combination with siRNA knockdown of ATR or ATM, revealed a synthetic lethal relationship between **1** and ATR that was not observed for ATM (Supplementary Figure 11). To establish a synergistic relationship, **1** was tested in combination with well-characterised ATR and ATM inhibitors VE-821[33] and KU-55933[35] (Figure 3B) in U2OS cells, and synergism determined by calculating drug combination indexes (CI, CompuSyn software). As single agents, **1** demonstrated an IC_{50} of 1.7 μM after 6 days in U2OS cells (measured using a cell proliferation MTS assay), whilst VE-821 and KU-55933 demonstrated IC_{50} values of 4.1 and 15.1 μM, respectively (Supplementary Figure 12). In combination, **1** demonstrated synergy with VE-821 (CI = 0.60) but not KU-55933 (CI = 1.41) (Figure 3C). This synergy is in agreement with previous siRNA studies that have shown depletion of USP4 impairs DNA repair, but does not affect the ATR-governed G2/M DNA damage checkpoint.[36] However, based on the multiple targets of **1** identified by ABPP we postulate that several factors are contributing to the observed effect, including the additional USP and redox process enzyme targets of **1**, the full characterisation of which requires further experiments.

In summary, we report a cell permeable ABP and its quantitative proteomic application to profile USP activity and target engagement in osteosarcoma cells that complements Ub-derived ABPs, which are limited to in-lysate profiling. **2** displays sub-micromolar labelling affinity towards 12 USPs, including several with clinical relevance or biomarker potential.[37] ABPP demonstrated that the parent compound **1** has good potency against USP4, USP16, and USP33 in U2OS cells; consistent with this profile, **1** was shown to functionally inhibit USP33, and to synergise

with ATR kinase inhibition. Potential use of derivatives of **1** are greatly hampered by the lack of selectivity of the compounds as well as their very limited plasma stability. Compound **2** establishes the potential for in-cell USP probes as a tool for target identification, and we anticipate that future generations of small molecule ABP will be useful in exploring a wide range of DUB biology in cells, as well as for competitive profiling against DUB inhibitors both *in vitro* and *in vivo*.

Methods

Chemical synthesis and experimental procedures are included in the supplementary information.

Associated content

Supporting Information Available: this material is available free of charge via the Internet. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE[38] partner repository with the dataset identifier PXD004875.

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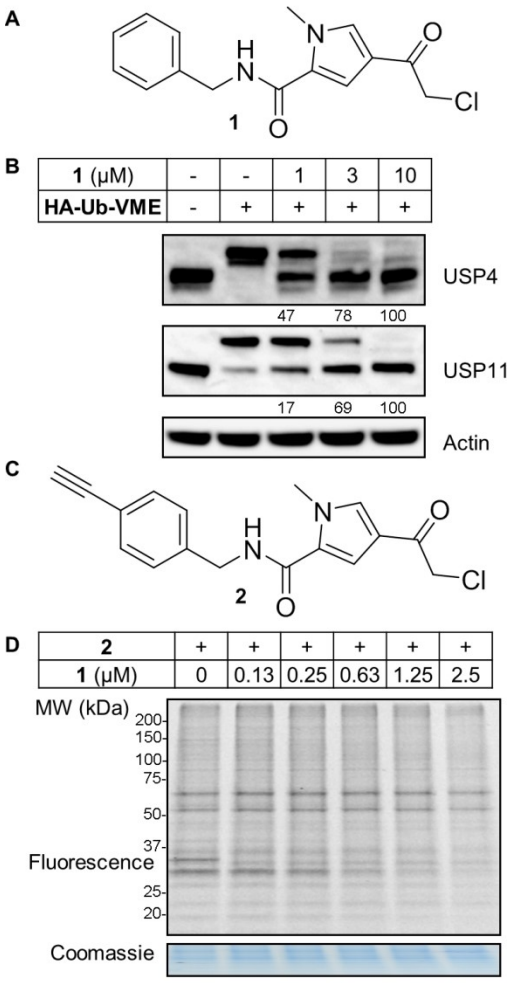


Figure 1. Compound **1** inhibits USP4 and USP11 and outcompetes labelling by compound **2** in intact U2OS cells. A) Structure of **1**. B) Western blot analysis of HA-Ub-VME ABP labelling of indicated DUBs following treatment with **1**. Inhibition was observed as a loss of (ABP-labelled) upper band intensity. Values represent percentage inhibition. C) Structure of **2**. D) In-gel fluorescence analysis of **2** (125 nM) labelled U2OS proteome in competition with **1**.

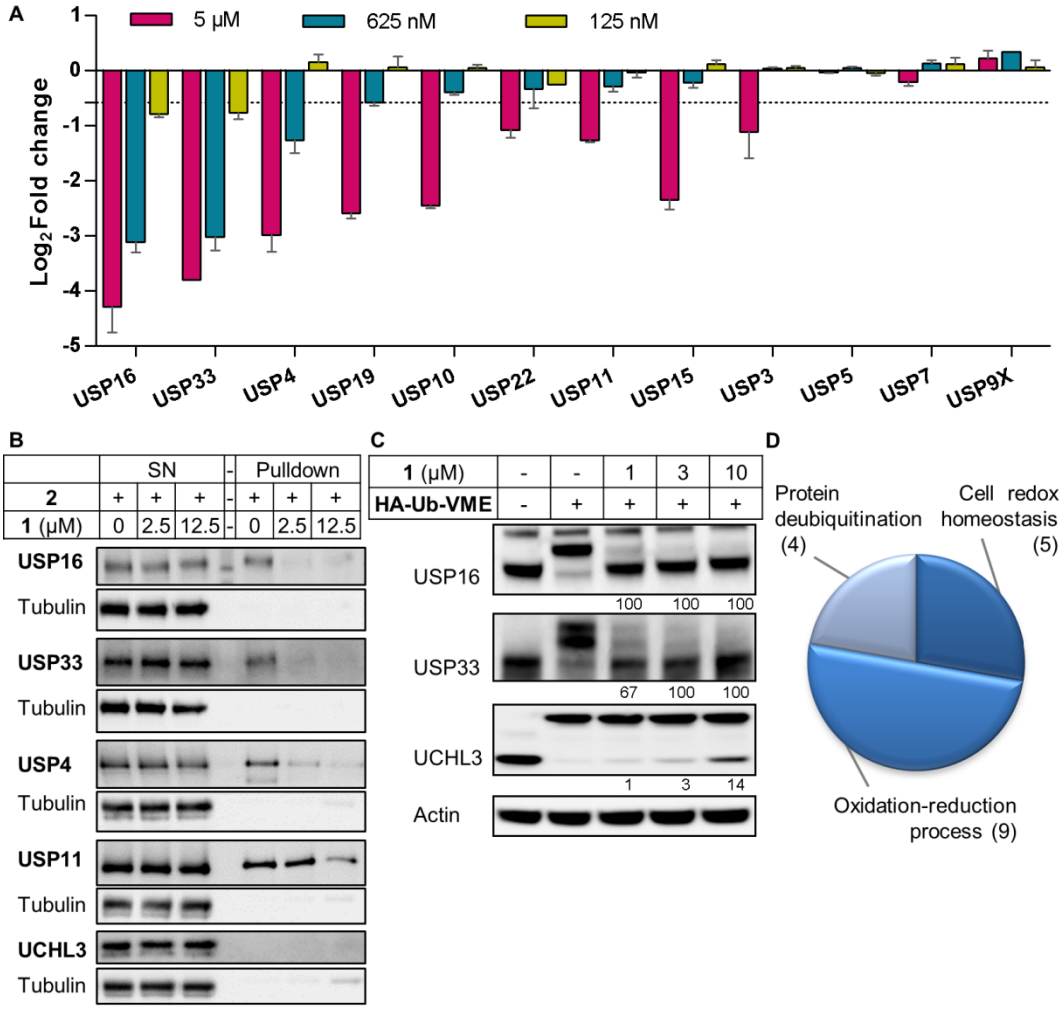
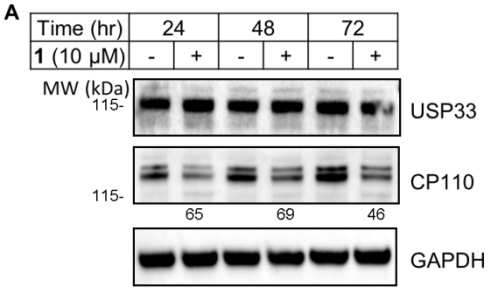


Figure 2. Compound **2** competitively labels USPs. A) Quantitative MS analysis of USP targets of **2** (125 nM) competed by **1** at indicated concentrations. The dashed line indicates a 1.5 fold change in ratio (log_2 = 0.58). B) Validation by immunoblotting of USP16, USP33, USP4 and USP11 competitive labelling and pulldown by **2** (2.5 µM). UCHL3 is included as a non-USP control. SN: supernatant. C) Target validation by immunoblotting HA-Ub-VME ABP labelling of indicated DUBs following treatment with **1**. Inhibition was observed as a loss of (ABP-labelled) upper band intensity. Values represent percentage inhibition. D) GO annotations (biological process) significantly enriched for the 27 targets of **2** (125 nM) competed by **1** (625 nM), obtained from STRING. The number of associated proteins is indicated.

Table 1. Biochemical IC_{50}s of **2** against recombinant DUB targets identified by quantitative MS analysis. Values were measured using an Ub-Rhodamine fluorescent intensity assay.

	Biochemical IC_{50} (μM)		**Biochemical IC_{50}** (μM)
USP4	0.05	USP15	1.71
USP11	0.21	USP10	2.72
USP16	0.14	USP7	44.2
USP19	0.35	USP5	>100

A



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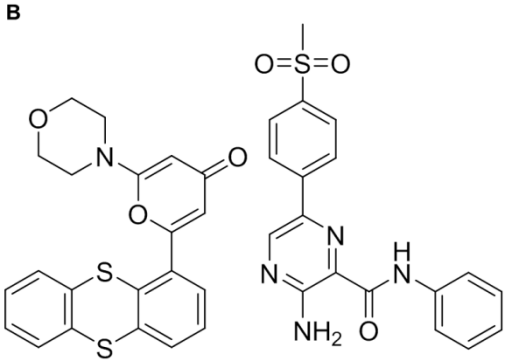

KU-55933 VE-821

C

Relative ratio: 1 : 10 : 3

1	KU-55933	VE-821	CI
+	+		1.41
	+	+	0.70
+		+	0.60
+	+	+	0.91

Figure 3. Compound **1** functionally inhibits USP33 leading to CP110 instability and synergises with ATR inhibition. A) Western blot analysis of **1**-treated U2OS cells. B) Structures of ATM inhibitor KU-55933 and ATR inhibitor VE-821. C) Chou-Talalay analysis of drug combination treatments. CI: combination index.