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Activity-Based Probes

Discovery of a Potent Deubiquitinase (DUB) Small-Molecule Activity-Based Probe Enables Broad Spectrum DUB Activity Profiling in Living Cells**

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Abstract: Deubiquitinases (DUBs) are a family of >100 proteases that hydrolyze isopeptide bonds linking ubiquitin to protein substrates, often leading to reduced substrate degradation through the ubiquitin proteasome system. Deregulation of DUB activity has been implicated in many diseases, including cancer, neurodegeneration and auto-inflammation, and several have been recognized as attractive targets for therapeutic intervention. Ubiquitin-derived covalent activity-based probes (ABPs) provide a powerful tool for DUB activity profiling, but their large recognition element impedes cellular permeability and presents an unmet need for small molecule ABPs which can account for regulation of DUB activity in intact cells or organisms. Here, through comprehensive chemoproteomic warhead profiling, we identify cyanopyrrolidine (CNPy) probe **IMP-2373** (**12**) as a small molecule pan-DUB ABP to monitor DUB activity in physiologically relevant live cells. Through proteomics and targeted assays, we demonstrate that **IMP-2373** quantitatively engages more than 35 DUBs across a range of non-toxic concentrations in diverse cell lines. We further demonstrate its application to quantification of changes in intracellular DUB activity during pharmacological inhibition and during MYC deregulation in a model of B cell lymphoma. **IMP-2373** thus offers a complementary tool to ubiquitin ABPs to monitor dynamic DUB activity in the context of disease-relevant phenotypes.

Introduction

The ubiquitin proteasome system (UPS) regulates myriad intracellular processes including protein turnover, transcriptional regulation, DNA damage, protein complex formation, cellular trafficking and localization, and inflammation and autophagy.[1] The E1/E2/E3 ligase cascade appends ubiquitin (Ub) to substrate proteins, a small post-translational modification which often tags proteins for degradation at the proteasome, although E3 ligase-mediated elongation of ubiquitin chains to various branched or linear forms can lead to diverse functional outcomes.[2–4] Approximately 110 individual deubiquitinase (DUB) proteases catalyze Ub hydrolysis from protein substrates or Ub chains, thereby counteracting Ub ligase activity and regulating the highly dynamic UPS. Altered DUB activity has been linked to a number of diseases and several DUBs are considered promising drug targets, with DUB inhibitors at various stages of preclinical or clinical development;[5,6] however, target validation for DUB inhibitors has proved challenging. For example, a recent phase I/II multiple myeloma trial of VLX1570, a putative USP14/UCHL5 covalent inhibitor, was terminated due to dose-limiting toxicity,[7] with subsequent proteomic analyses revealing crosslinking of a diverse range

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of proteins and off-target toxicity through protein aggregation.[8] There is a pressing need for improved chemical tools and technologies to better understand DUB abundance, localization, activity and substrate profiles in health and disease, and to support development of novel, effective and selective DUB-targeted therapeutics.[9]

DUB activity-based probes (ABPs) represent a uniquely powerful tool for exploring changes in cellular activity, and are based on an electrophilic warhead targeted to the DUB active site by a recognition scaffold, with DUB activity read out by a reporter group such as a dye or affinity handle.[10] The majority of DUBs contain papain-class cysteine peptidase active sites amenable to covalent labeling by an appropriately designed ABP.[5,6] The first generation of DUB ABPs based on ubiquitin as the recognition element (Ub ABPs)[11] have served to monitor DUB proteolytic activity and substrates in disease states,[12] Ub chain cleavage selectivity,[13] and DUB inhibitor potency and selectivity.[14] Whilst the 8.5 kDa Ub recognition element makes extensive interactions with the DUB and thereby delivers specific DUB enrichment from complex biological media, very poor cellular uptake restricts their effective use to analysis of cell lysates (Figure 1A). The consequent loss of native organelle compartmentalization leads to dilution of DUB concentration and dissociation of protein-protein interactions (PPIs) involved in DUB activity.[15] The disconnect between enzyme activity in lysates and live cells is well-recognized,[6] and places limits on the capacity of Ub ABPs to profile dynamic intracellular DUB activity or its role in a particular disease state.[16] Ub ABP uptake can be forced by high concentration and conjugation to cell-penetrating peptides (CPPs),[15] but these complex approaches further disrupt cell membrane integrity and are not generally applicable to diverse cell lines, primary cells or animal models.[17]

Small molecule DUB ABPs with broad in-family DUB reactivity, which can passively diffuse into cells with minimal perturbation to cell physiology, have the potential to complement Ub ABPs by profiling intracellular DUB activity or inhibition across many DUBs simultaneously. Two types of small molecule DUB ABP with intracellular labeling activity have been reported to date: highly targeted cyanopyrrolidine (CNPy) probes for the DUB UCHL1 (e.g. **IMP-1710**),[17–20] and the pan-reactive chloromethyl ketone (CMK) pyrrole benzylamide probe (**4**).[21] ABP **4** was shown to engage at least nine Ubiquitin-Specific Proteases (USPs) by proteomics, and could be used to measure USP4 activity in live osteosarcoma cells. Whilst this probe offered a promising proof of concept, it lacks pan-DUB coverage and in-class selectivity, and its limited DUB specificity leads to considerable toxicity at concentrations useful for profiling. We envisaged expanding the scope of this small molecule scaffold to engage the active DUB proteome (or DUBome) at scale, with sufficient potency and selectivity to permit activity profiling without toxicity. Here we designed and extensively profiled a library of probes based on this scaffold covering a diverse range of warhead reactivities and electrophile geometries, exploring intracellular protein labeling, cell viability and DUB target engagement and activity profiles. These screens led to the discovery of next generation, pan-active, cell permeable DUB ABP **IMP-2373** (**12**), bearing a cyanopyrrolidine (CNPy) warhead, exhibiting privileged DUB labeling and selectivity across a range of

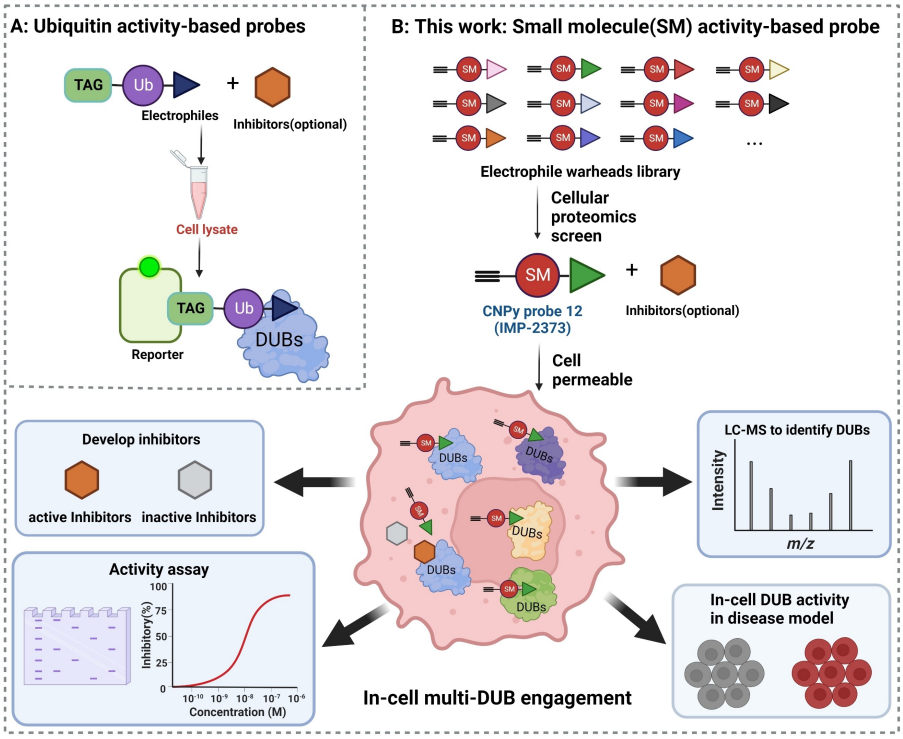


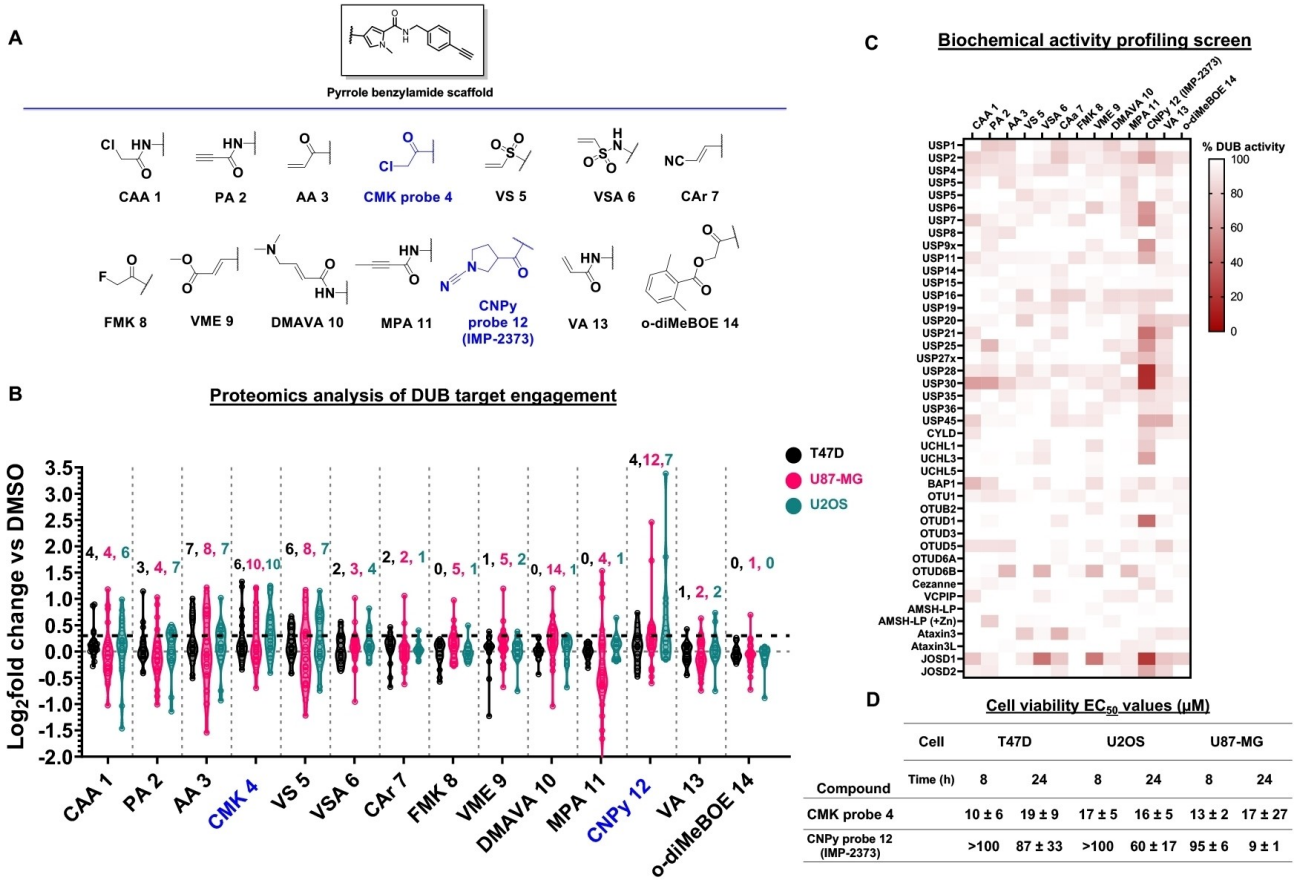
Figure 1. Pipeline of activity-based probe applications. (A). Ubiquitin activity-based probes; (B) Small molecule activity-based probe.

cell lines and disease models. We show that **IMP-2373** (**12**) represents a novel and versatile small molecule tool for probing DUB biology in complex physiological systems, which complement existing Ub ABPs (Figure 1B).

Results and Discussion

Chemical proteomic profiling of small molecule electrophilic warheads uncovers cyanopyrrolidine as a privileged DUB-targeting moiety

We first designed and synthesized a library of fourteen pyrrole benzylamide probes displaying diverse cysteine reactive electrophiles (Figure 2A), including previously reported CMK probe **4**, selected to reflect a range of reactivity and geometric diversity whilst maintaining synthetic tractability.[22] Osteosarcoma (U2OS), glioblastoma (U87-MG) and breast cancer (T47D) cell lines were selected for initial probe screening experiments, as these widely-used lines together express >80 % of all DUBs, as measured by mRNA profiles (Figure S1). Live cells from each cell line were incubated with each probe for proteome-wide target engagement profiles determined by multiplexed quantitative Tandem Mass Tag (TMT) Activity-Based Protein Profiling (ABPP) at 3 μM (Figure 2B). As reported previously, CMK probe **4** exhibited broad DUB target engagement with 14 DUBs enriched by $\log_2$ fold change >0.3 compared with vehicle (DMSO) (Figure S3A). Interestingly, CNPy probe **12** exhibited the most consistent DUB target engagement ($\log_2$ fold change >0.3 vs DMSO) among all the probes (Figure 2B), with a broadly complementary profile to CMK probe **4** (Figure S3A). These data suggested that CNPy may be a privileged DUB ABP warhead, consistent with several reports on CNPy covalent DUB inhibitors.[19,20,23–25]



Compound	Time (h)	T47D 8	T47D 24	U2OS 8	U2OS 24	U87-MG 8	U87-MG 24
CMK probe 4		10 ± 6	19 ± 9	17 ± 5	16 ± 5	13 ± 2	17 ± 27
CNPy probe 12 (IMP-2373)		>100	87 ± 33	>100	60 ± 17	95 ± 6	9 ± 1

Figure 2. Quantitative proteomic activity-based protein profiling of a series of electrophile-armed methyl pyrroles in three cell lines identifies cyanopyrrolidine (CNPy) probe **IMP-2373** (**12**) as a multi-DUB targeting probe. (A) Synthesized chemical structures of cysteine reactive electrophile warhead library. (B) Proteomics analysis of DUB target engagement obtained by ABPP (numbers represent the number of DUBs quantitatively engaged $\log_2$ fold change >0.3 vs vehicle (DMSO) in each data set) for the compound library at 3 μM incubation concentrations for 1.5 h in each of three cell lines (T47D, U2OS, U87-MG). (C) Cyanopyrrolidine ABP (CNPy probe **12**) engages a wide range of DUBs in biochemical activity profiling (D) Cell viability measured by EthD-1 and Calcein AM dual dye cell death assay of CNPy probe **12** (**IMP-2373**) and CMK probe **4** in T47D, U2OS, and U87-MG cells.

Cyanopyrrolidine probe IMP-2373 (12) is a potent broad-spectrum DUB enzyme inhibitor with minimal off-target cytotoxicity

We next compared this indicative assessment of cellular activity and selectivity against capacity to inhibit enzyme activity across a panel of 42 recombinant DUBs (Figure 2C). Strikingly, CNPy probe **12** displayed a markedly superior profile with respect to potency and promiscuity of DUB inhibition over all other warheads tested, similar to that observed by proteomic profiling, and complementary to that previously reported for CMK probe **4** (Figure S3B).[21] Furthermore, despite its promising DUB engagement profile (Figure 2B), **4** exhibits cytotoxic effects within a few hours at 1 μM which are likely due to the high electrophilicity and promiscuous reactivity of the CMK warhead, confounding its use as a DUB ABP which should ideally show minimal impact on cell physiology at concentrations sufficient for measurable probe engagement (Figure 2D, S4A–F).[21] Conversely, CNPy probe **12** did not affect cell viability at 8 h treatment, and remained tolerated up to 50 μM after 24 h, as measured by two distinct cell death assays, such as the ethidium homodimer (EthD-1), Calcein AM dual dye (Figure 2D, S4A–C), and a Sytox Green time-resolved cell death imaging (Figure S4D–F), with the exception of U87-MG cells, which may be due in part to the strong dependence of gliomas on UCHL1 activity for proliferation.[26] Competition experiments with Ub-VME suggested inhibition of multiple DUBs in cell lysates, consistent with activity-based binding of CNPy probe **12** (Figure S5A), which was prioritized for further experiments and renamed **IMP-2373** to support future reference beyond the present study.

CNPy IMP-2373 is a broad-spectrum ABP for a significant proportion of the DUBome

Encouraged by the low cytotoxicity of **IMP-2373**, we explored probe concentration and an extended incubation time (4 h) to optimize DUB engagement (Figure S5B). A total of 28 DUBs were enriched over DMSO control (log_2 fold change >0.5 vs DMSO) at one or more concentrations tested by activity-based protein profiling (Figure 3A and C). Furthermore, enrichment of 20 DUBs engaged by a

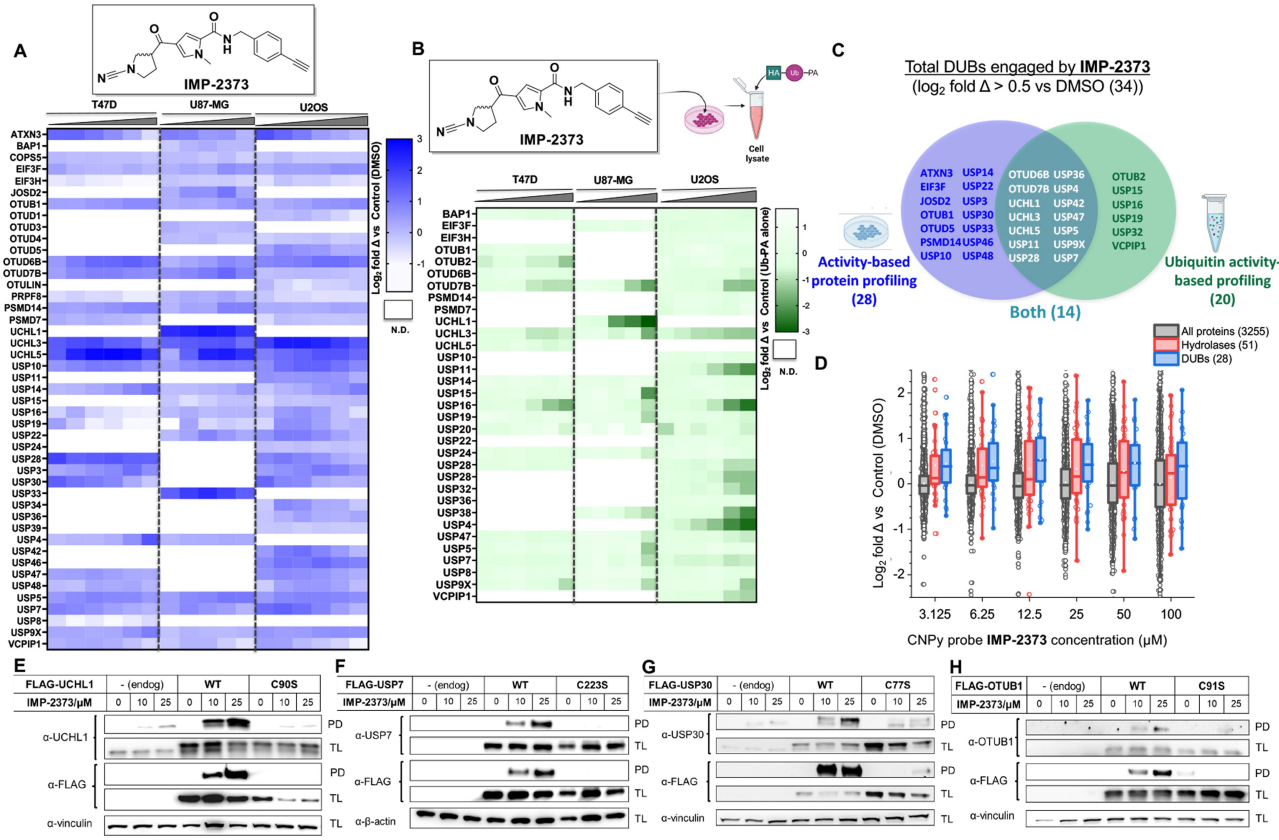


Figure 3. (A) Higher CNPy probe **IMP-2373** concentrations and a longer treatment time (4 h) allows for activity-based profiling of >35% of the DUBome. Heat maps for activity-based protein profiling (ABPP), and competitive ubiquitin activity-based profiling (Ub-ABP) (B) CNPy probe **IMP-2373** (concentrations: 3.125, 6.25, 12.5, 25, 50 and 100 μM, 4 h treatment, 37 °C, 5% CO_2) in 3 cell lines. N.D.—not detected. (C) CNPy probe IMP-2373 inhibits the activity—of 36 DUBs (log_2 fold change >0.5 vs DMSO or <−0.5 vs Ub-ABP) across all sub-classes (with the exception of ZUP1) by ABPP or Ub-ABP competition. (D) In T47D cells, DUBs were preferentially enriched over all other proteins, and over hydrolases in general, the parent enzyme class of DUBs. HEK293T cells were transfected to overexpress FLAG-tagged WT UCHL1 (E), USP30(F), USP7(G), OTUB1(H) or catalytic CS mutants, and pulldown following treatment with **IMP-2373** for 1 h. (PD: pulldown, TL: total lysate). The corresponding workflow and uncropped images of Figure 3E–H are shown in Figure S7A–E in the Supporting Information.

standard HA (human influenza hemagglutinin peptide)-tagged ubiquitin propargyl amide (HA-Ub-PA) ABP could be outcompeted by **IMP-2373** ($\log_2$ fold change < -0.5, negative for competition) (Figure 3B and C).

14 DUBs were identified as hits in both studies, consistent with activity-based engagement by **IMP-2373** at the DUB active site (Figure 3C). Statistical analysis of relative protein abundance after probe enrichment between 10 μM and 25 μM suggested that DUBs were differentially enriched not only relative to all proteins identified, but also relative to hydrolases in general, the parent enzyme class which encompasses DUBs (Figure 3D). A total of approximately 70 non-DUB proteins were enriched over control under at least one of the concentrations tested, with only eight non-DUB proteins enriched consistently across the three cell lines (Figure S6A–D); gene ontology analysis confirmed that these off-targets are also primarily peptidases and related enzymes (Figure S6E). To confirm dependence on DUB catalytic activity for cellular target engagement, we expressed a series of diverse FLAG-tagged wild-type (WT) DUBs (UCHL1, USP30, USP7 and OTUB1) across three DUB subfamilies and corresponding active site Cys to Ser mutants (CS) in HEK293T cells (Figure S7A). Western blot analysis of affinity-enriched DUB following 1 hour treatment with 0, 10 or 25 μM **IMP-2373** suggested that the probe selectively engaged the catalytic cysteine of the tested DUBs (Figure 3E–H, Figure S7A–E). Since the CNPy warhead features a stereogenic center, we were interested to explore whether the enantiomers showed a preference for different DUB subclasses. We separated the enantiomers by chiral chromatography and profiled each by ABPP and Ub-ABP proteomics in T47D cells (Figure S8A). Interestingly, statistical analysis suggested that one of the enantiomers exhibited slightly more potent DUB enrichment at low concentration (0.5 μM, Figure S8B) with a trend towards selectivity observed only for the UCH DUB subfamily, consistent with previous reports on stereoselectivity in CNPy UCHL1 ABPs (Figure S8C–D).[19]

We next explored the capacity of **IMP-2373** to act as a competitive probe for in-cell target engagement by selective DUB inhibitors, a powerful and useful application of ABPs in drug discovery and development.[8,27] Cells were pre-treated for 1 h with increasing concentrations of a selective active site inhibitor for UCHL1 (**IMP-1711-S**)[19] or USP30 (**FT385**),[28] followed by 10 μM **IMP-2373** for 1 h. Competitive activity-based profiling (Figure S9A–C) by pull-down and western blot analysis confirmed potent and selective concentration-dependent in-cell target engagement for each inhibitor (Figure 4A and 4B, S9D–E). No competition was observed at any concentration for **IMP-1711-R**, the inactive enantiomer of the UHCL1 inhibitor (Figure 4A, S9D), consistent with robust activity-based profiling.[19]

CNPy ABP IMP-2373 enables differential DUB activity profiling during MYC deregulation in a B cell lymphoma model

To demonstrate the potential of **IMP-2373** as a chemical tool to monitor changes in DUB activity in disease models, we turned to a widely-used model of MYC deregulation in cancer.[29] MYC is a multifunctional transcription factor which regulates expression of a large number of genes involved in cellular growth, proliferation and metabolism.[30] MYC deregulation can lead to dramatically altered protein synthesis by driving massive increases in gene transcription, and enhanced production of ribosomes and translation initiation factors,[32] promoting cell growth, cell cycle progression, and genome instability, ultimately leading to oncogenesis and malignant tumor growth. Aberrant MYC is an oncogenic driver in $>50\,\%$ of human cancers, and the mechanisms by which MYC-deregulated cancers cope with radically altered protein turnover may present novel therapeutic targets.[30]

Multiple DUBs have been proposed to regulate MYC ubiquitination and stability, including USP13,[31] USP29,[32] OTUB1.[33] Here, we applied **IMP-2373** to test the hypothesis that MYC drives dynamic changes in DUB activity as part of the adaptation of cancer cells to deregulated protein synthesis, employing human lymphoblastoid B cell line P493-6, in which conditional MYC expression is under the control of an inducible promoter.[29] P493-6 cells constitutively express c-MYC (high-MYC), however addition of doxycycline and β-estradiol for 72 h potently downregulates expression, resulting in a low-MYC state (Figure 4C, S10). Cells in each state were exposed to a short treatment with **IMP-2373**, (25 μM, 1 h), and whole proteome and activity-based proteomic analyses undertaken to enable differential quantification of overall protein expression and probe labeling in high- vs low-MYC cells, for each probe-treated or vehicle-treated condition (Figure 4D). **IMP-2373** captured the activity of 38 DUBs across MYC high and low cells (Figure S11–S14), and this broad coverage of DUBs by both ABPP and quantitative whole proteome analysis allowed direct comparisons between DUB abundance and activity across high- and low-MYC cell lines.

Whilst the abundance of most DUBs showed only small changes between MYC states, global DUB activity was markedly lower in high-MYC cells (Figure S13A–B, S14A), and changes in DUB activity and abundance were essentially uncoupled (Figure S14B). Statistically significant upregulation of activity relative to abundance was observed in low-MYC cells for multiple DUBs, including UCHL3, USP7, USP47, USP10 and ATXN3, offering the first insights into MYC-dependent differential regulation of DUB activity in intact cells (Figure 4E, S13B).

ABPs are well-established for certain enzyme classes, such as fluorophosphonate ABPs for serine hydrolases.[34,35] A similar "in-family pan-reactive" ABP for DUBs could enable detection of changes in enzyme activity in response to specific cellular perturbations, and elucidation of how DUBs drive particular cellular phenotypes.[36] In this work, we introduce CNPy probe **IMP-2373** as a versatile in-cell DUB ABP, with applications including inhibitor profiling and quantification of DUB activity changes in a biological context. DUB substrate selectivity is typically driven by extensive macromolecular interactions, presenting a significant challenge for small molecule DUB probe design which must retain small size to avoid cell impermeability, as is the

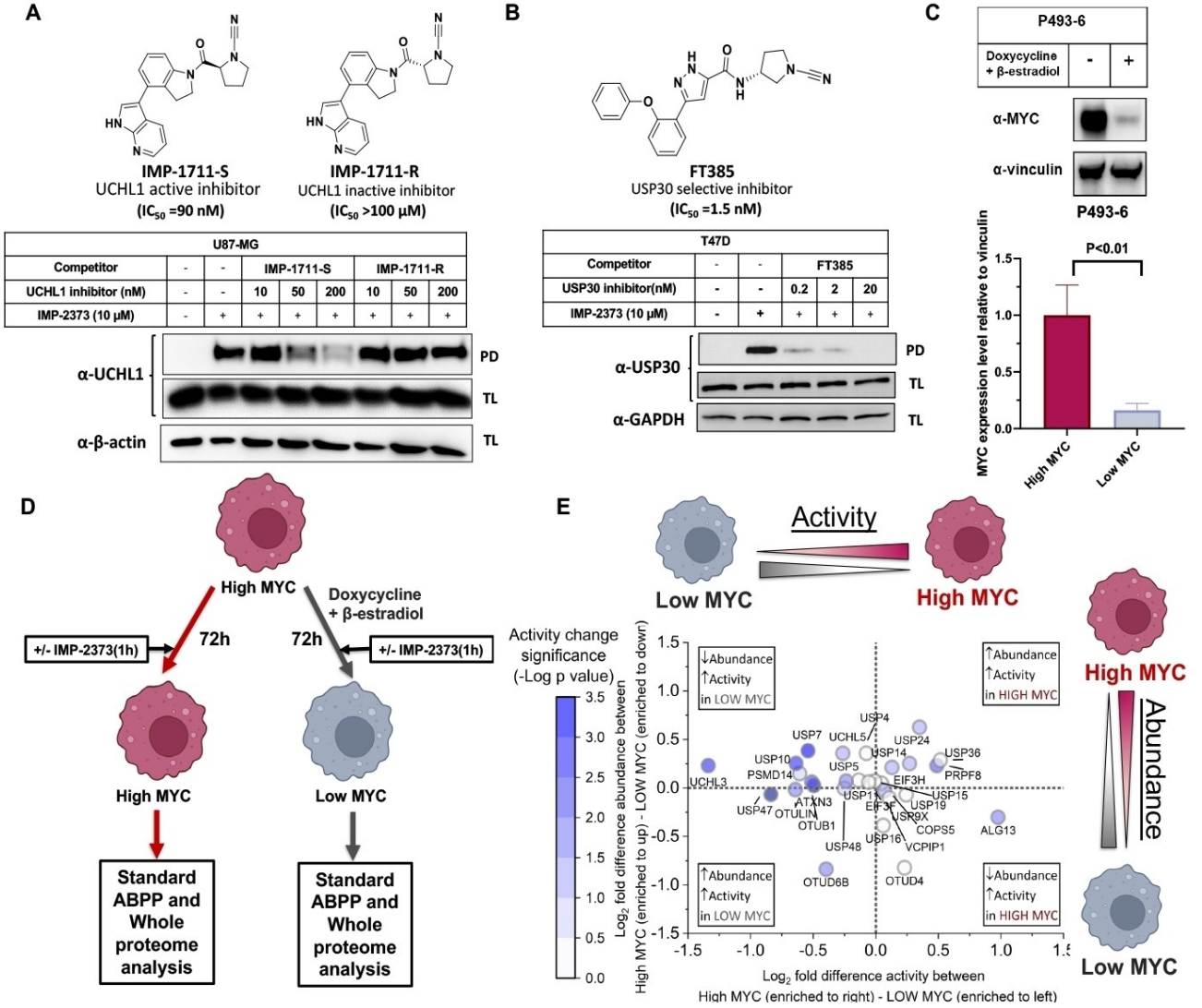


Figure 4. Cyanopyrrolidine ABP **IMP-2373** can be applied to profile inhibitors and to monitor DUB activity in response to differential MYC expression. Target engagement of either compound **IMP-1711-S** (UCHL1 active inhibitor), **IMP-1711-R** (UCHL1 inactive inhibitor) (A), or **FT385** (USP30 selective inhibitor) (B) was captured by competition ABPP with CNPy probe **IMP-2373**. TL—Total Lysate; PD-Pull down. The corresponding workflow and uncropped images of Figure 4A–B are shown in Figure S9A–E in the Supporting Information. (C) western blot validation of statistically significant MYC protein level reduction in P493-6 cells in response to treatment with doxycycline and β-estradiol for 72 h. The replicates images of Figure 4C are shown in Figure S10 in the Supporting Information. (D) Experimental design to detect changes in DUB activity in response to MYC deregulation. (E) Statistically significant differences in DUB activity and abundance between low and high levels of MYC, as measured by ABPP and whole proteome profiling with and without CNPy ABP **IMP-2373** (25 μM, 1 h, $n=3$).

case for 8.5 kDa Ub ABPs. Covalent capture of the DUB active enzyme site with an electrophilic warhead offers a potentially powerful approach for potent and selective DUB inhibition, however challenges remain in tuning such warheads towards DUBs. Several studies point to cyanopyrrolidines as a privileged warhead for DUBs, with enhanced reactivity toward the DUB active site relative to other classes of protease, including a recent report suggesting that this warhead may organize the DUB catalytic site in UCHL1.[17,19,20,24,25,37]

CNPy probe **IMP-2373** exhibits a clear preference for DUBs over other proteins in general, and even within the hydrolase class, resulting in greatly reduced cytotoxicity compared to previous designs. However, whilst labeling can be readily achieved at sub-toxic concentrations in multiple cell lines, **IMP-2373** retains residual labeling activity at eight conserved off-target proteins (Figure S6D) which may perturb cellular phenotype. Additionally, whilst competition against a Ub-ABP (Figure 3B) and competition against inhibitors (Figure 4A, 4B) indicate robust dose-responsive labeling by **IMP-2373**, ABPP experiments with probe alone suggests variable and even counter-intuitive inverse dose responses for some DUBs (Figure 3A). The cause of this phenomenon remains unclear, but it may be in part due to changes in DUB turnover triggered by high target occupancy, or probe-activated cellular stress pathways.[38] Cau-

tion should therefore be exercised when selecting concentration and incubation time for in-cell probe applications. Our comprehensive study suggests that the working concentration range of the probe should not exceed 50 μM, and that one-hour treatment with ca. 10–25 μM probe is sufficient to capture most DUB activities. However lower probe concentrations may permit capture of specific highly engaged DUB subsets, such as the UCHL family. Future probe optimization could be achieved by mining the rapidly growing biochemical CNPy DUB inhibitor patent literature[23–25,39–42] coupled with systematic docking analyses across known and predicted human DUB structures (e.g. AlphaFold)[43] to optimize the scaffold attached to the CNPy warhead for DUBs over off-target proteins, or for a particular DUB subfamily. In conjunction with conventional structure-based design and optimization, this approach may eventually permit discovery of even more potent and selective DUB-privileged small molecules and ABPs for future fundamental biology and therapeutic application. The present probe design requires two-step labeling, and although this adds an additional click ligation step and requires removal of excess reagents through precipitation prior to enrichment, the small alkyne handle minimizes negative impacts on probe physicochemical properties and cell uptake.[44]

To our knowledge, the present ABPP experiments using **IMP-2373** in intact B cell lymphoma cells provide the first evidence for dynamic changes in DUB activity uncoupled from changes in abundance, as a result of oncogenic MYC deregulation. Interestingly, our results also suggest that MYC deregulation may provoke downregulation of multiple DUB activities, which we hypothesize is consistent with adaptation to increased protein translation in these cells,[31] as it will tend to upregulate ubiquitination, protein degradation and protein turnover. In future it will be interesting to explore potentially cytotoxic activation of UCHL3, USP7, USP47, USP10 or ATXN3 in MYC-deregulated cancers, and to investigate the substrate profiles of these enzymes in MYC-deregulated cells. Further to this, we suggest that **IMP-2373** could be applied to understand changes in DUB activity in other pathological and physiological processes featuring rapid changes in protein turnover, such as the switch to cellular quiescence[45] or senescence in cancer,[46] UPS-independent but Ub-dependent degradation pathways such as autophagy or mitophagy,[47] or host-pathogen interactions, for example during infection by viruses which hijack endogenous DUB activity.[48]

Conclusion

By exploring a diverse range of electrophiles attached to a constant 4-methylpyrrole benzylamide scaffold, we have identified cyanopyrrolidine as a privileged warhead for broad spectrum ABPs targeting DUB enzymes. CNPy probe **IMP-2373** represents the most potent and selective pan-DUB small molecule ABP reported to date and permits profiling of DUB activities and DUB inhibitors in intact cells, providing a useful complement to Ub ABPs in studies to uncover regulation of DUB activity.

Abbreviations

DUB	Deubiquitinase
Ub	ubiquitin
UPS	ubiquitin proteasome system
ABP	activity-based probe
Ub-ABP	Ub-activity-based probe
CNPy	cyanopyrrolidine

Supporting Information

Supplementary figures for additional experimental results (Figures S1–14) .pdf. Additional experimental details, materials, and methods .pdf. ^{1}H NMR spectra of final chemical probes.pdf. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE[49] partner repository with the dataset identifier PXD035417.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Conflict of Interest

Jaimeen D. Majmudar, Christopher W. am Ende, Dafydd Owen, Monica Schenone, Dahye Kang, Liang Xue, Sheila Kantesaria and Linda Lohr are/were employees of Pfizer during the execution of this work. Edward W. Tate is a founding director and shareholder of Myricx Pharma Ltd., an advisor of and holds share options in Sasmara Therapeutics and receives current or recent funding from Myricx Pharma Ltd, Pfizer Ltd, Kura Oncology, AstraZeneca, Merck & Co., GSK.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords: Chemical Probe · Deubiquitinase · Electrophilic Warhead · MYC-Deregulated Cancer · Ubiquitin Activity-Based Probe

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