

# Re-evaluating the mechanism of action of $\alpha,\beta$ -unsaturated carbonyl DUB inhibitors b-AP15 and VLX1570: a paradigmatic example of unspecific protein crosslinking with Michael acceptor motif-containing drugs.

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## Abstract

Deubiquitinating enzymes are a growing target class across multiple disease states, with several inhibitors now reported. b-AP15 and VLX1570 are two structurally related USP14/UCH-37 inhibitors with a shared  $\alpha,\beta$ -unsaturated carbonyl substructure motif. Initially taken forward into a phase I/II clinical trial for refractory multiple myeloma, VLX1570 has since been put on full clinical hold due to dose limiting toxicity. Through a proteomic approach, here we demonstrate that these compounds target a diverse range of proteins, resulting in the formation of higher molecular weight complexes. Activity-based proteome profiling identified CIAPIN1 as a sub-micromolar covalent target of VLX1570, and further analysis demonstrated that high molecular weight complex formation leads to aggregation of CIAPIN1 in intact cells. Our results suggest that in addition to DUB inhibition, these compounds induce non-specific protein aggregation through cross-linking, providing a molecular explanation for general cellular toxicity.

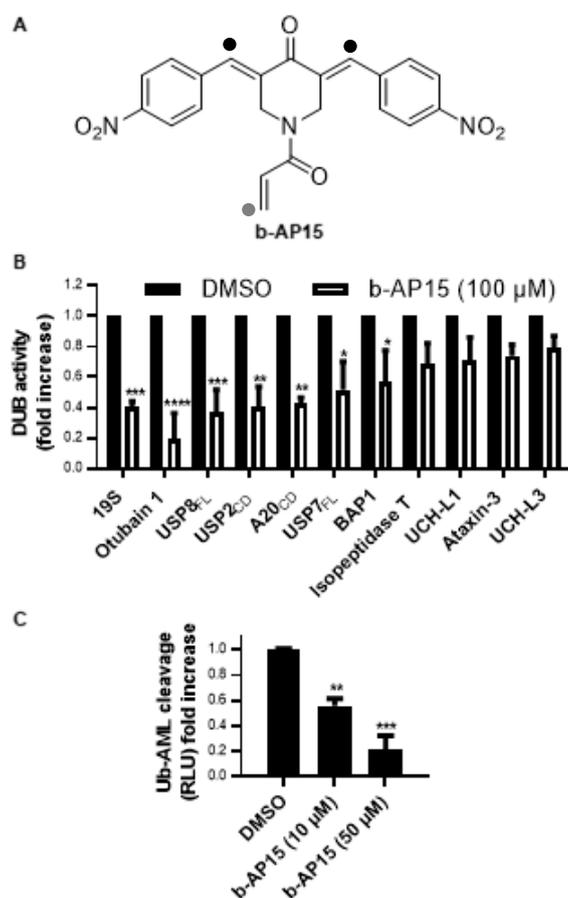
## Main

Ubiquitination, the covalent addition of the 76 amino acid protein ubiquitin (Ub) to protein substrates, is a widespread protein post-translational modification (PTM) in eukaryotic cells.<sup>1</sup> Due to its role in proteasomal degradation and a plethora of signalling pathways, ubiquitination is an emerging field of clinical interest in multiple

disease states, including cancer.<sup>2-4</sup> Several compounds have been reported in recent years targeting deubiquitinating enzymes (DUBs)<sup>4, 5</sup> which regulate the removal of Ub marks. However, the characterisation of such inhibitors and clinical compounds from a target perspective is variable; though a DUB target is reported, compound selectivity and specificity across the proteome remained in most cases incompletely resolved. Comprehensive understanding of a compound's targets facilitates interpretation of phenotypes in pre-clinical investigations, and can identify mechanisms of toxicity<sup>6</sup> and resistance at an early stage of testing. Only recently, and with the help of advanced activity-based protein profiling (ABPP)<sup>7-9</sup> assays that allow the profiling of DUBs in a cellular context, highly selective DUB inhibitors have been reported.<sup>4, 5, 10</sup> Here, we describe the proteomic investigation of two structurally related DUB inhibitors b-AP15 and VLX1570. We demonstrate that these inhibitors target a diverse range of proteins, resulting in the formation of higher molecular weight (MW) complexes. Through a quantitative chemical proteomic approach, we identify CIAPIN1, also known as anamorsin, as a potent covalent target of VLX1570 which upon reaction with VLX1570 forms high molecular weight complexes leading to aggregation of CIAPIN1 in intact cells.

b-AP15 has been previously described as a specific reversible inhibitor of the proteasomal DUBs USP14 and UCH-37 (also referred to as UCH-L5) with anti-cancer activities.<sup>11, 12</sup> Examination of the chemical structure of b-AP15, however, shows the presence of Michael acceptor motifs (**Figure 1A**) that are capable of covalent interaction with nucleophilic residues. Although the results obtained by D'Arcy and colleagues demonstrate that b-AP15 is an inhibitor of USP14/UCH-37,<sup>11</sup> two unrelated cysteine protease enzymes from different DUB families, its structure suggests additional proteins may be targeted by this compound class. Indeed, b-AP15 possesses higher potency in intact cells than in biophysical assays against USP14 and UCH-37.<sup>11</sup> In support of compound promiscuity, another study describing the chemical synthesis of active-site-directed DUB probes showed data compatible with a non-specific DUB inhibition profile upon increasing concentrations of b-AP15.<sup>13</sup> In our hands, b-AP15 inhibits the cleavage of the DUB substrate Ub-AML (ubiquitin-aminoluciferin) by a number of purified recombinant DUBs (**Figure 1B**). Crude extracts of cells treated with increasing concentrations of b-AP15 also

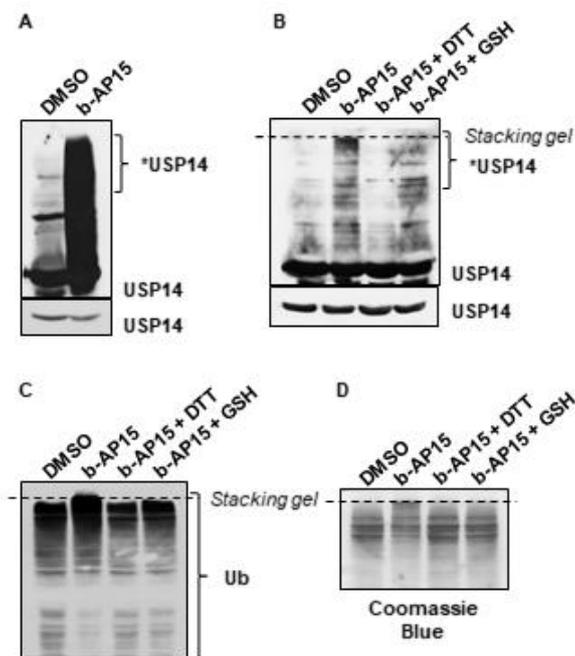
showed that b-AP15 is able to decrease the global DUB activity of these treated cells (**Figure 1C**). Further, b-AP15 treatment in both cancer cell lines and endothelial cells resulted in comparable cytotoxicity as observed by a cell viability assay, indicating the non-specific toxicity of this chemotype (**Supplementary Figure 1**).



**Figure 1. b-AP15 is a non-specific deubiquitinating enzyme (DUB) inhibitor.** (A) Molecular structure of b-AP15 with Michael acceptors (black dot) and acrylamide (grey dot) motif indicated. (B) DUB activity measured by cleavage of the luminescent substrate Ub-AML in a panel of recombinant proteasomal (purified 19S proteasomes) and non-proteasomal DUBs treated with b-AP15 (100 μM) (n=3-4). (C) DUB assay as described above using HeLa cell extracts (5 μg) incubated with the indicated b-AP15 concentrations (n=3).

By immunoblot analysis, it was observed that b-AP15 induces the formation of high MW complexes with USP14 (**Figure 2A and 2B**) and UCH-37 (**Supplementary Figure 2**) in both crude cell extracts and intact cells. The formation of these complexes is reduced by co-incubation with thiol containing reducing reagents DTT or GSH, in support of these higher MW complexes forming via the Michael acceptors. The protective effect of these reagents was hypothesized to be due to the blocking of these reactive sites. To test this, b-AP15 was reacted *in vitro* with an

excess of GSH in PBS, representing conditions similar to those used in our *in vitro* and *in situ* assays. As predicted, b-AP15(GSH)<sub>2</sub> could be observed by LC-MS analysis after 1 hour of incubation. (**Supplementary Figure 3**). In support of this finding, PYR-41, an ubiquitin-activating enzyme (E1) inhibitor (also containing Michael acceptors), has been reported to induce DTT-sensitive protein cross-linking and inhibition of DUBs and other cellular enzymes by formation of high MW complexes<sup>14</sup>. In addition, high MW ubiquitylated proteins accumulated upon b-AP15 treatment, the levels of which were reduced by co-treatment with DTT and GSH (**Figure 2C**). The origin of these very high molecular weight ubiquitylated proteins that accumulate upon treatment with b-AP15 in comparison with those that accumulate on application of proteasome inhibitors has been largely discussed.<sup>11, 15</sup> Interestingly, ubiquitylated protein complexes with very high MW are also reported by D'Arcy and other studies using  $\alpha,\beta$  unsaturated carbonyl compounds.<sup>14, 16-18</sup> The effects of b-AP15 on the global proteome were further evaluated by SDS-PAGE followed by Coomassie Blue staining, revealing the formation of notably increased levels of high MW protein complexes in comparison to DMSO control or co-treatment with DTT and GSH (**Figure 2D**). To better characterize the consequences of the global formation of high MW protein complexes on cell signalling, the status of mTOR was analysed in crude extracts treated with b-AP15 by immunoblotting. Strikingly, a shift from mTOR in its native form towards a band with slower migration mobility is observed with nanomolar concentration of bAP-15 (**Supplementary Figure 4A and 4B**). A similar observation was described by us in a previous study showing oxidation of proteins by a photosensitiser.<sup>19</sup> A similar but less potent effect was observed on incubation with the natural product curcumin, which also possesses Michael acceptor groups (**Supplementary Figure 4C and 4D**).



**Figure 2. b-AP15 inhibits DUBs through the formation of cytotoxic high MW protein complexes, which are averted in the presence of thiol reducing agents.** Immunoblot analysis of high MW complexes of USP14 in (A) HeLa crude cell extracts incubated with b-AP15 (10  $\mu$ M) and in (B) HeLa cells treated with either vehicle, 10  $\mu$ M b-AP15 or with 10  $\mu$ M b-AP15 in combination with reducing agents (10 mM dithiothreitol (DTT) or 10 mM glutathione (GSH)), both at 37°C for 2 hours. Shorter exposure times were used as a loading control (lower blots in the panel). (C) Immunoblot analysis of ubiquitin conjugates in HeLa cells treated with vehicle, 10  $\mu$ M b-AP15, or with 10  $\mu$ M b-AP15 in combination with reducing agents (10 mM dithiothreitol (DTT) or 10 mM glutathione (GSH)) for 2 hours. (D) Visualization of the b-AP15-induced high MW protein complexes in a SDS-PAGE gel, after Coomassie Blue staining, in HeLa cells treated as in (C).

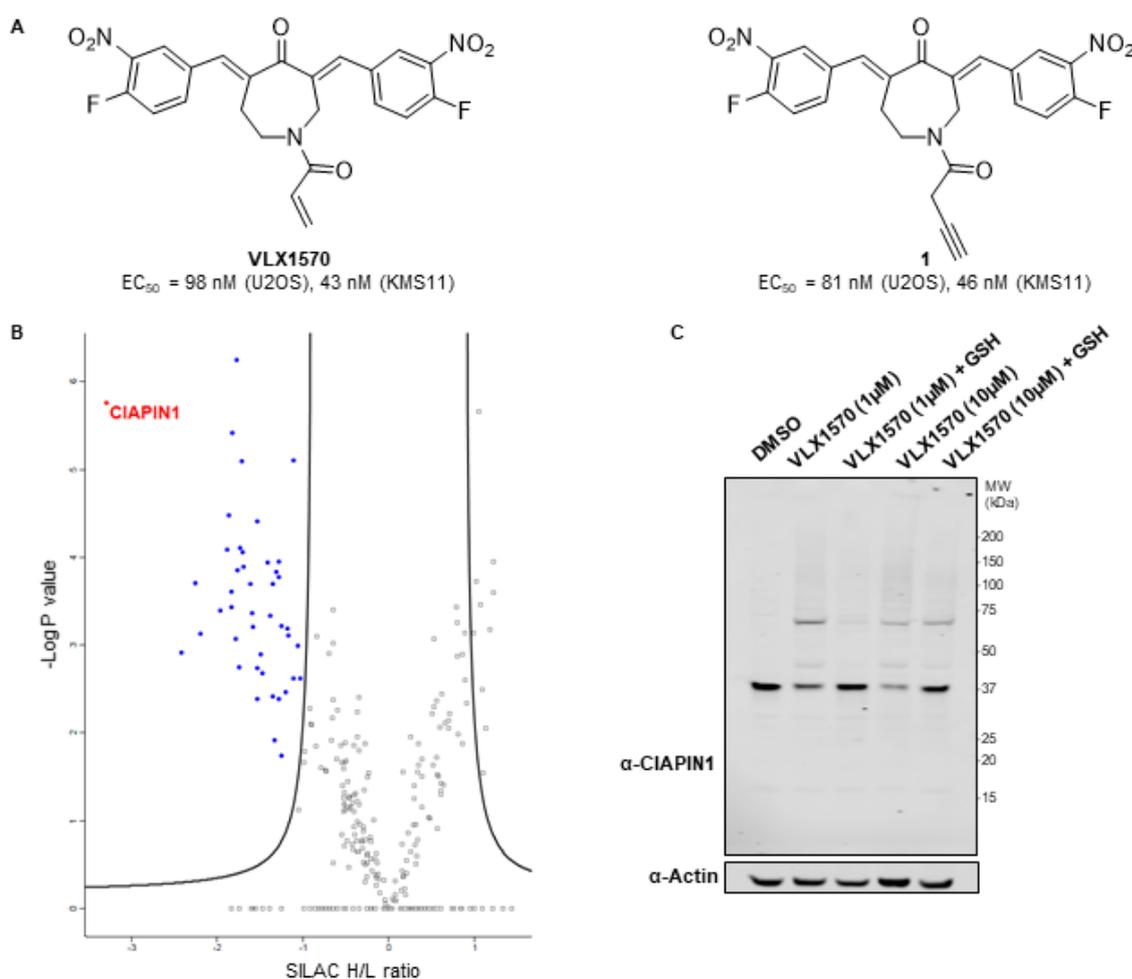
VLX1570 is a structural analogue of b-AP-15 that shows higher potency and improved permeability.<sup>20, 21</sup> VLX1570 was taken forward into a phase I/II clinical trial for refractory multiple myeloma in combination with dexamethasone (NCT02372240), but was then put on full clinical hold in July 2017 due to dose limiting toxicity. Based on our analysis of b-AP15, we postulated that this toxicity may be due to promiscuous covalent reaction with cellular nucleophiles and formation of high MW complexes. To test whether VLX1570 also forms high MW complexes, HeLa lysates were treated with varying concentrations of b-AP15 and VLX1570 and probed with the active-site-directed DUB probe HA-Ub-C2Br.<sup>22</sup> Dose-dependent inhibition of probe labelling was observed for both compounds. Even on blotting for USP28, a DUB structurally unrelated to both USP14 and UCH-37, higher MW complex formation was observed (**Supplementary Figure 5**). Co-incubation of both VLX1570 and b-AP15 with GSH in the multiple myeloma cell line KMS11 led to increased cell

viability after 16 hours, suggesting that the GSH is scavenging these compounds and reducing their active concentration (**Supplementary Figure 6**).

GSH represents one of many nucleophiles accessible to VLX1570 within a biological context, and Michael acceptors are well known for their reactivity with cysteine residues.<sup>23</sup> To comprehensively profile the covalent targets of VLX1570, ABPP was conducted. Accordingly, an alkyne tagged analogue of VLX1570, Compound **1** (**Figure 3A**) was designed and synthesized. The acrylamide position was selected for conversion to the alkyne, as acrylamide-lacking analogues had previously been reported in structure-activity relationship studies not to influence cell viability.<sup>20</sup> Indeed, **1** retained equivalent cytotoxicity to VLX1570 in both KMS11 and U2OS cell lines, as observed with the CellTiter Glo assay (**Figure 3A, Supplementary Figure 7**).

Before commencing proteomic-profiling experiments, suitable competitive conditions were determined. U2OS cells were pre-incubated with increasing concentrations of VLX1570 or bAP-15 for 30 minutes before addition of the affinity probe **1** at a final concentration of 5  $\mu$ M. After 1 hour, the cells were lysed. Subsequent ligation to AzTB,<sup>24</sup> an azido-TAMRA-biotin capture reagent, via a copper catalyzed azide-alkyne cycloaddition (CuAAC) allowed visualization of probe-protein complexes by in-gel fluorescence. Both VLX1570 and b-AP15 pre-incubation caused a reduction in labelling intensity for specific bands, most markedly affecting a band at approximately 37 kDa (**Supplementary Figure 8A**). 5 and 20  $\mu$ M VLX1570 competitive conditions were taken forward for proteomic analysis. Spike-in SILAC methodology<sup>25</sup> was employed as previously described.<sup>8, 26</sup> Briefly, cell lysates from competitive conditions were mixed in a 2:1 ratio with 'spike': lysate generated from **1**-treated U2OS cells grown in R10K8 media. Subsequent CuAAC ligation, enrichment on NeutrAvidin-Agarose resin, tryptic digest and LC-MS/MS analysis enabled proteome-wide in-cell target identification of **1**-labelled proteins. Multiple covalent targets were identified using this method, with significant competition observed at both 1- and 4-fold competitor excess. 44 proteins were significantly competed by 20  $\mu$ M VLX1570 (**Figure 3B, Supporting Dataset 1**), 24 of which were also significantly competed by 5  $\mu$ M of compound (**Supplementary Figure 8B**). On inspection of these protein sequences, all contained cysteine residues. Gene Ontology cellular component analysis suggested that VLX1570 was covalently interacting with proteins across multiple cellular locations, including the cytoplasm,

nucleus, and multiple organelles (**Supplementary Table 1**). It should be noted that although we have demonstrated accumulation of higher MW bands for UCH-37, USP14 and USP28 by immunoblot analysis, none of these DUBs were identified in the ABPP experiment. As the ABPP experiment only identifies covalent interactors, it is possible that the high MW complex formation observed for DUBs is occurring through a non-covalent mechanism. This is in agreement with literature data reporting that although DTT does affect cytotoxicity of VLX1570<sup>20</sup>, compound binding and enzymatic inhibition are both reversible.<sup>21</sup> Alternatively, it is possible that the high MW complexes formed with these proteins sterically hinder the CuAAC ligation reaction, thereby preventing covalently **1**-labelled proteins from being enriched and identified in this experiment.



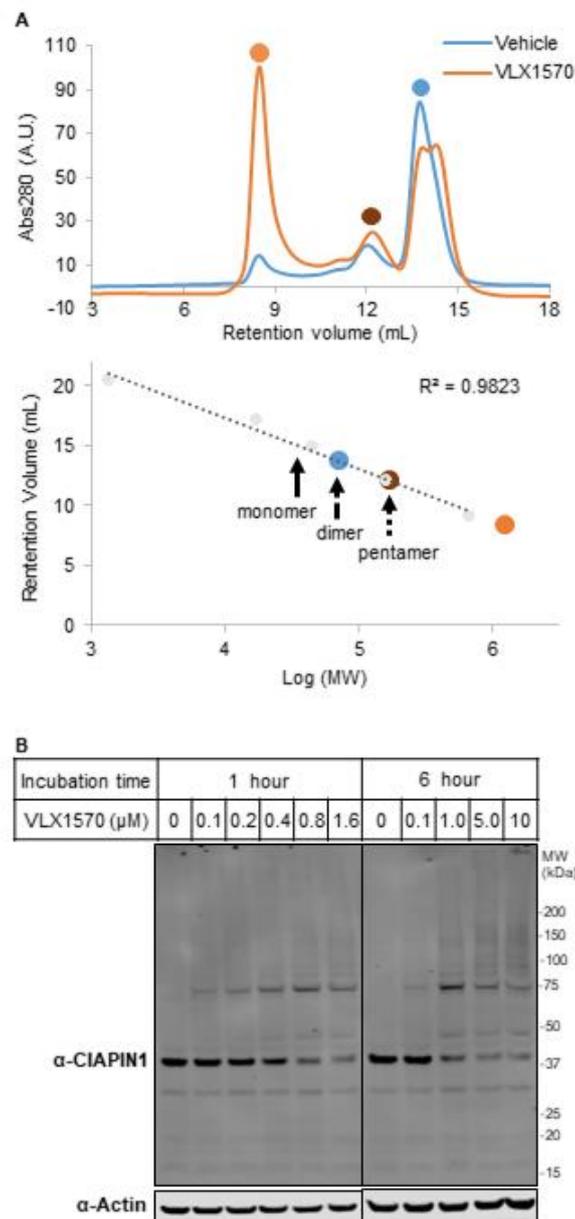
**Figure 3. Chemical proteomics reveals multiple covalent targets of VLX1570.** (A) Structure of VLX1570 and probe derivative compound **1**. EC<sub>50</sub> values measured by CellTiter Glo in U2OS and KMS11 cells are reported. (B) Volcano plot of **1**-enriched proteome from U2OS cells, with targets significantly (FDR=0.05, S<sub>0</sub> = 0.2) competed by 20 µM VLX1570 indicated (blue). The most significant

hit, CIAPIN1, is indicated. **(C)** Immunoblot analysis of VLX1570 treated KMS11 cells demonstrates a protective effect against high MW complex formation for CIAPIN1 on addition of GSH (10 mM, 2 hr).

The most significantly competed covalent target of VLX1570 identified was CIAPIN1. CIAPIN1, a 33 kDa protein, is a part of the electron transport chain that enables Fe-S cluster assembly.<sup>27</sup> It possesses an N-methyltransferase domain at its N-terminus, though this has been reported to be catalytically inactive.<sup>28</sup> Further, CIAPIN1 exerts anti-apoptotic effects in cells though it is unrelated to apoptosis regulatory molecules of the BCL2 or CASP families.<sup>29</sup> In our hands, siRNA knockdown of CIAPIN1 lead to a reduction in cell viability of KMS11 cells (**Supplementary Figure 9**). Knockdown of CIAPIN1 has been reported to induce apoptosis in several cancer<sup>30, 31</sup> and also non-cancer cell lines.<sup>32, 33</sup> In agreement with functional inhibition of CIAPIN1, both b-AP15 and VLX1570 are reported to exert cytotoxicity via an apoptotic mechanism that is insensitive to BCL2 overexpression.<sup>11, 21</sup>

As observed for DUB targets of this chemotype, higher MW complex formation was observed on immunoblotting for CIAPIN1 in VLX1570 treated cells, an effect that was rescued on addition of GSH (**Figure 3C**). Incubation of VLX1570 with recombinant CIAPIN1 showed similar higher MW complex formation (**Supplementary Figure 10A**) and formation of a covalent adduct was confirmed by electrospray mass spectrometry (MS) analysis (**Supplementary Figure 10B**). Further analysis by size exclusion chromatography demonstrated that these VLX1570 adducts result in the accumulation of protein aggregates (**Figure 4A**). LC-MS/MS analysis of the CIAPIN1-VLX1570 adduct following tryptic digest revealed that 7 out of a possible 10 cysteine residues were covalently modified by VLX1570, indicating that though VLX1570 modifies CIAPIN1 monomerically, it does so non-specifically (**Supplementary Figure 10C**). Closer inspection of an unmodified peptide containing Cys249 showed the presence of an intra-chain disulfide bond, which was disrupted in the equivalent VLX1570 modified peptide (**Supplementary Figure 11, Supplementary Table 2**). This disrupted disulfide bond represents one potential mechanism by which VLX1570 adducts result in CIAPIN1 instability and ultimately aggregation. Finally, we examined the fate of CIAPIN1 in intact cells following VLX1570 treatment. VLX1570 was titrated onto KMS11 cells and incubated for 1 or 6 hours before lysis and immunoblot analysis (**Figure 4B**). Aggregation was observed with 100 nM of VLX1570 after 1 hour, with

extended higher MW complexes and depletion of stable CIAPIN1 observed after 6 hours. This effect was conserved for b-AP15 (**Supplementary Figure 12**).



**Figure 4. VLX1570 aggregates CIAPIN1.** (A) Size exclusion chromatography of recombinant CIAPIN1 in the presence of VLX1570 or DMSO control indicates formation of an aggregation product. (B) Immunoblot analysis of high MW complex formation of CIAPIN1 following incubation with indicated concentrations of VLX1570 or DMSO

In summary, our data indicate that b-AP15 and VLX1570 react with multiple cellular proteins in a non-specific manner. Through a combination of immunoblot analysis and ABPP we have identified several additional DUB targets, and multiple proteins

unrelated to the ubiquitination machinery. Toxicity observed for drug candidates has been reported to be caused by off-target effects.<sup>34</sup> In the case of b-AP15 and VLX1570, we show that it is linked to the formation of high MW complexes following compound treatment, an effect that is abated by co-incubation with GSH. Other molecules like curcumin<sup>18</sup> and its derivative AC17<sup>16</sup>, the diterpene NSC-302979<sup>18</sup>, synthetic compounds DBA<sup>18</sup> and PYR-41<sup>14</sup>, the prostaglandin  $\Delta$ 12-PGJ<sub>2</sub><sup>18</sup>, and the chalcone derivatives AM146<sup>17</sup>, RA-9<sup>17</sup> and RA-14<sup>17</sup>, all contain  $\alpha,\beta$ -unsaturated ketones and have been already described to inhibit isopeptidases. The affinity probe **1** has enabled the comprehensive profiling of the covalent targets of VLX1570 in intact cells, leading to the identification of CIAPIN1 as a target. Further analysis of CIAPIN1 has identified protein depletion through aggregation as a mode of action of VLX1570, supporting our observations with b-AP15.

Collectively, the data demonstrate the power of target profiling to elucidate mechanism of action and potential toxicity early in compound development. This strikes a cautionary note for the application of structurally similar compounds as tool compounds, and the clinical investigation of compounds possessing this chemotype.

## Methods

Chemical synthesis and experimental procedures are included in the Supporting Information.

## Data availability

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE<sup>35</sup> partner repository with the dataset identifier PXD015412. Reviewers may access the data using the following credentials: Username: [reviewer39460@ebi.ac.uk](mailto:reviewer39460@ebi.ac.uk); Password: 7Prr05Lq

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