On Terminal Alkynes That Can React with Active-Site Cysteine Nucleophiles in Proteases

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Ⓢ *Supporting Information*

ABSTRACT: Active-site directed probes are powerful in studies of enzymatic function. We report an active-site directed probe based on a warhead so far considered unreactive. By replacing the C-terminal carboxylate of ubiquitin (Ub) with an alkyne functionality, a selective reaction with the active-site cysteine residue of de-ubiquitinating enzymes was observed. The resulting product was shown to be a quaternary vinyl thioether, as determined by X-ray crystallography. Proteomic analysis of proteins bound to an immobilized Ub alkyne probe confirmed the selectivity toward de-ubiquitinating enzymes. The observed reactivity is not just restricted to propargylated Ub, as highlighted by the selective reaction between caspase-1 (interleukin converting enzyme) and a propargylated peptide derived from IL-1β, a caspase-1 substrate.

Figure 1. (A) Ubiquitin functionalized with propargylamine replacing Gly76. (B) Fluorescence polarization-based substrate turnover assay measuring UCHL3 activity, showing Ub-Prg as 10^5 times more powerful an inhibitor than Ub. Dotted line represents the concentration of UCHL3 (60 pM) used. (C) Deconvoluted mass of UCHL3 (calcd monoisotopic mass, 26 166 Da) before/after reaction with Ub-Prg. A mass increase of 8544 Da is observed, corresponding to one Ub-Prg molecule.

Ubiquitination is among the most abundant post-translational protein modifications. Ubiquitin (Ub), a 76 amino acid protein, can be covalently linked through its C-terminal carboxylate to the ε-amine of a lysine residue or the N-terminus of a target protein. This process can be reversed by the action of de-ubiquitinating enzymes (DUBs). Ubiquitination and de-ubiquitination are important in cellular homeostasis and signaling.[1] As DUBs are involved in a host of cell biological processes, they constitute an attractive therapeutic target.[2]

C-terminally propargylated Ub (Ub-Prg, Figure 1A) was synthesized, using a previously reported linear solid-phase peptide synthesis procedure.[3] We generated Ub-Prg as a substrate for triazole-linked peptide-Ub[4] conjugations using click chemistry.[5] In a fluorescence polarization-based DUB activity assay,[6] Ub-Prg inhibited the human DUB, ubiquitin carboxyl-terminal hydrolase isozyme L3 (UCHL3), with approximately equimolar stoichiometry (Figure 1B). This finding was surprising, as terminal alkynes are considered inert under physiological conditions.[7] LC-MS (Figure 1C) and SDS-PAGE analysis (Figure S1) revealed that Ub-Prg forms a covalent bond with UCHL3 that is resistant to denaturing conditions. This reaction could be abolished by inhibition of UCHL3 with
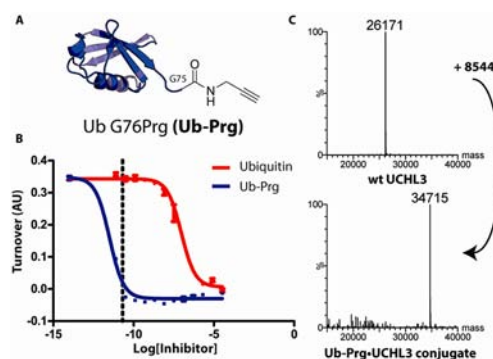
N-ethylmaleimide, a cysteine alkylating reagent, prior to addition of Ub-Prg. In contrast, a 1000-fold excess of propargylamine, *N*-glycinylpropargylamide, or free thiol (DTT, β-mercaptoethanol, or glutathione) over UCHL3 did not affect the reaction outcome (Figure S1). Titration of Ub-Prg against UCHL3 confirmed the 1:1 reaction stoichiometry (Figure S2).

To determine the reaction rate, we performed an *in vitro* time course experiment (Figure S3). UCHL3 showed quantitative reaction with Ub-Prg within 1 min, similar to the rate previously reported for the Ub-based DUB-probe, Ub vinyl methyl ester (Ub-VME).[8] Reaction between UCHL3 and Ub-Prg yielded a product equal in mass to the sum of both reactants. The acid lability of the purified UCHL3·Ub-Prg complex (Figure S4) suggested the formation of a vinyl thioether linkage. The nature of the linkage formed was confirmed by solving the crystal structure of a DUB in complex with Ub-Prg. The viral ovarian
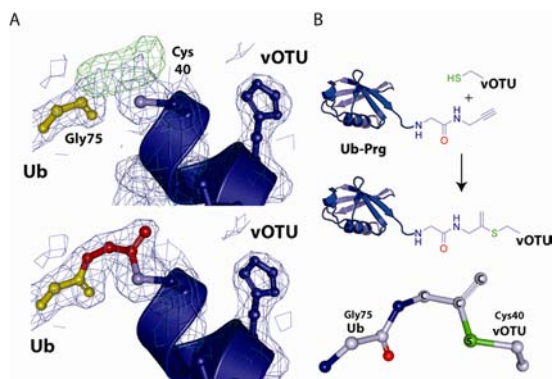
Figure 2. Structure of vOTU (blue) in complex with Ub-Prg (yellow). (A) Electron density maps (blue represents $2|F_o| - |F_c|$ contoured at 1σ; green represents $|F_o| - |F_c|$ at 3σ) covering the catalytic Cys of vOTU and the C-terminus of Ub-Prg, before (top) and after refinement (bottom) with the vinyl thioether linker. (B) Reaction between vOTU and Ub-Prg (top) and representation of the reaction product as observed by X-ray crystallography (bottom), showing the vinyl thioether linkage in the Ub-Prg complexed structure.

Figure 3. SDS-PAGE gel showing reaction of Ub-Prg derivatives. R_1 = N-terminally TMR-labeled Ub_{1-75}, R_2 = unlabeled Ub_{1-75}, and R_3 = Ub_{1-74}. Only probes 6, 7, and Ub-Prg show significant reactivity toward UCHL3.

tumor DUB (vOTU) encoded by Crimean Congo hemorrhagic fever virus (CCHFV)[9] was reacted with Ub-Prg. The resulting complex was crystallized and its structure determined at 2.3 Å resolution (Figure 2A, Table S1). The refined structure closely resembles previously determined vOTU·Ub complexes[10] (rmsd = 0.4–0.6 Å^2). Refinement of the complex structure excluding the propargyl group at the C-terminus of Ub yielded positive difference electron density ($|F_o| - |F_c|$) connecting Gly75 to the catalytic Cys40 residue of vOTU (Figure 2A). The electron density obtained unambiguously revealed the attachment of the Cys thiol atom to the quaternary carbon in Ub-Prg (Figure 2B), confirming a vinyl thioether linkage.

We assessed the scope and limitations of this reaction by studying the reaction between UCHL3 and a series of Ub-Prg analogues (Figure 3). An allylic variant **1** of the alkyne showed minor reactivity. Ub-propyl, with (**2**) or without (**4**) an N-terminal tetramethylrhodamine (TMR) label, did not react with UCHL3. The terminally methylated alkyne (2-butynyl) derivative **3** failed to react, suggesting an important role for the terminal CH proton. A 2,2-dimethylated variant (2-methyl-3-butyn-2-amine-modified, **5**) did not react with UCHL3, suggesting either steric hindrance around the C2-atom being inhibitory or an essential role for a proton at this position. The amide group β to the alkyne moiety did not contribute to the reaction, as a but-3-ynyl analogue (**6**) reacted equally well. An analogue, with the last two glycine residues replaced by a hex-5-ynyl-1-amine (**7**) to afford an isostere of Ub-Prg, reacted as well, albeit at a lower rate. This is likely due to the absence of the H-bonding amide linkage between Gly75 and Gly76 and UCHL3, which contribute to binding affinity. Together these data suggest that the mildly electron-withdrawing effect of the amide bond is not essential. Based on these observations, we consider three possible mechanisms for this reaction: (1) direct nucleophilic attack on the quaternary propargyl carbon; (2) formation of an allene followed by nucleophilic or radical attack; and (3) formation of a thiol radical in the enzyme active site followed by radical reaction with the quaternary carbon center of the alkyne.

Radical-mediated reactions between thiols and alkynes have been described.[11] In thiol-yne reactions, alkynes can undergo a double thiol addition resulting in a dithioether. Sterically hindered alkynes or thiols on the other hand can undergo a
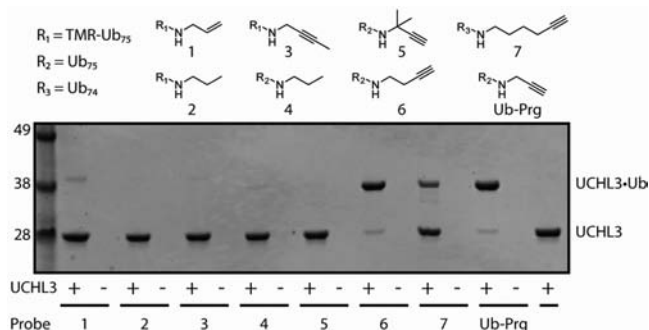
single addition, resulting in a vinyl thioether.[12] To study the involvement of a radical intermediate in the reaction, we reacted UCHL3 and Ub-Prg in the presence of NO-generating reagents[13] (NONOates, Figure S5), as NO is a small, potent, and water-soluble radical scavenger. Pre-incubation of Ub-Prg with NO for 30 min followed by incubation with UCHL3 did not affect the reaction outcome, nor did addition of a different scavenger, galvinoxyl free radical.[14] These data together do not support a radical mechanism.

Formation of an allene intermediate in solution prior to binding the enzyme is unlikely, as the reaction proceeds under acidic conditions (Figure S6), and strong bases are usually required for allene formation. The establishment of an allenic equilibrium in the active site followed by nucleophilic attack by the active-site cysteine cannot be excluded based on the current data.

We next analyzed whether the reaction was restricted to UCHL3 or other classes of DUBs could react with Ub-Prg as well. We found Ub-Prg to react with recombinant members of all four families of cysteine DUBs (Figures 4A and S7), including A20, a notoriously unreactive member of the OTU-DUB family that does not react with other Ub-based active site probes.[15] Other classes of cysteine proteases, including the protease for the Ub-like modifier SUMO, SENP6, did not react with Ub-Prg, nor did Ub-activating enzyme E1 (Figure S7).

Fluorescent activity-based probes[16] accelerate the process of activity-based protein profiling.[17] To determine whether Ub-Prg could be used as a general fluorescent activity-based profiling reagent, we incubated a TMR-labeled version of Ub-Prg (TMR-Ub-Prg) with a panel of GFP-labeled recombinant DUBs in MelJuso cell lysates. A fluorescence gel scan (Figure S8) showed that cysteine DUBs could be labeled in lysates. Western blot analysis (Figure 4B) showed band shifts corresponding to one Ub moiety, compared to unlabeled DUBs. Mutation of the active-site cysteine residue to serine abolished DUB labeling. TMR-Ub-Prg could also be used to label native DUBs in cell lysates (Figure 4B,C).

This ability to label DUBs in lysates was compared to that of the commonly used probe Ub-VME. The above panel of DUBs expressed in MelJuso cells was labeled under identical conditions with Ub-Prg or Ub-VME (Figure S9). While Ub-VME reacted with some but not all tested DUBs in this assay, Ub-Prg modified all active DUBs tested. Differences in labeling between Ub-Prg and Ub-VME were further studied by pre-incubating EL-4 lysate with unlabeled Ub-Prg and Ub-VME, respectively. After depletion of DUBs, the remaining active DUBs were visualized
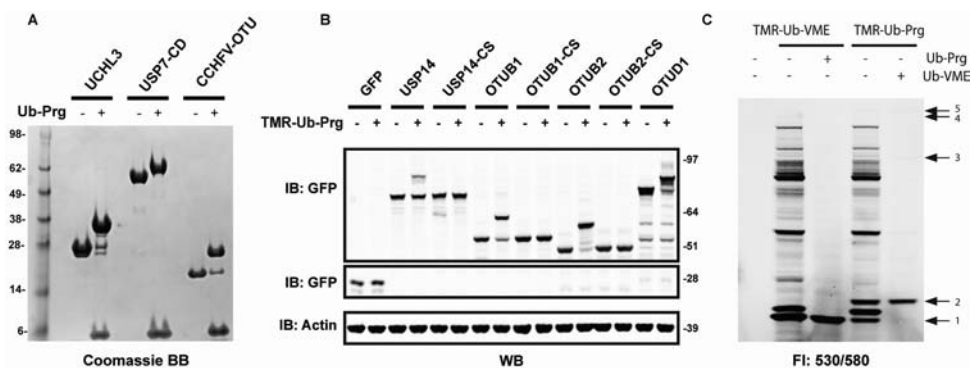
Figure 4. (A) SDS-PAGE analysis of *in vitro* reaction of three different classes of DUBs with Ub-Prg. (B) GFP fusions of DUBs from the USP and OTU-clades were transfected in MelJuSo cells, and their reaction with Ub-Prg was visualized using anti-GFP Western blot. DUBs annotated with -CS are catalytic cysteine-to-serine mutants. For images of direct fluorescence scans see Figure S8. (C) Comparison of DUB labeling between TMR-Ub-VME and TMR-Ub-Prg. Third and fifth lanes are pretreated with unlabeled Ub-Prg and Ub-VME, respectively. Labeled lysates were analyzed using SDS-PAGE, and tagged DUBs were visualized by fluorescence scanning. An overexposed fluorescent image showing DUBs 3−5 is available as Figure S10.

with a fluorescently labeled competitor probe, showing four unique DUBs labeled by Ub-Prg, after Ub-VME depletion, indicated by arrows (Figures 4C and S10).

The stability of the propargyl moiety allowed direct immobilization of Ub-Prg on CNBr-activated sepharose resin (Figure S11). This resin was used to verify the selectivity of Ub-Prg for DUBs and its breadth of reactivity across the different DUB families in cell lysates. The binding capacity of the resin was tested by incubating decreasing amounts of resin with a known amount of UCHL3 in the presence of cysteine-rich bovine serum albumin (Figure S12). This allowed easy separation of UCHL3 from albumin. Using this methodology, the loading of the resin was determined to be 0.4−0.7 μmol/g of resin. We then used the resin to covalently capture active DUBs from cell lysates of the mouse lymphoma cell line (EL-4), as a benchmark cell line for the study of active-site directed DUB probes.[12] The covalent attachment of DUBs to immobilized Ub-Prg allowed for very stringent denaturing washing conditions to remove nonspecifically bound proteins. To release the isolated DUBs from the resin, we applied the lability of vinyl thioethers to strong acidic conditions (Figure S12).[18] This allowed for SDS-PAGE analysis of the pulldown products prior to trypsinolysis. Samples could also be analyzed directly using on-bead trypsinolysis followed by LC-MS/MS.

Identification of the isolated DUBs by LC-MS/MS (Figures 5 and S13) showed that members of all four known classes of
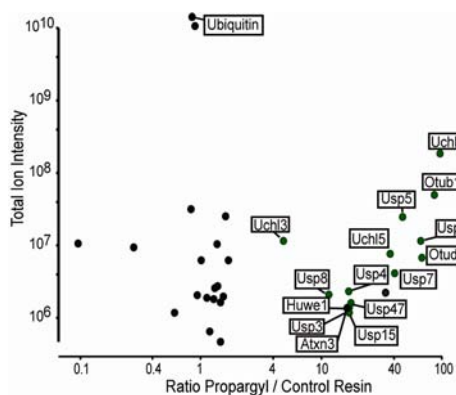
cysteine DUBs were retrieved in a single simple experiment. Using a dimethyl-labeling-based quantitative proteomics approach,[19] all recovered DUBs displayed a selective enrichment over background (Figure 5). For all recovered DUBs, with the exception of UCHL3, no signal was observed in the control sample. Nonspecific interactors were not found to be significantly enriched. Only the E3-ligase HUWE1 was found, which has also been reported using other active-site directed probes.[20] The general applicability of this reaction was tested on another member of the family of Ub-like proteins, Nedd8 (Figure S14). A synthetic propargylated Nedd8 reacted efficiently with UCHL3, which is known to cross-react with Nedd8.[21]

To test whether reaction of alkynes with active site cysteine nucleophiles is limited to the class of Ub-like proteases or can be applied to other families of cysteine proteases as well, we synthesized propargyl analogues of common peptide aldehyde-based cysteine protease inhibitors by directly converting the aldehyde to alkyne using Bestmann−Ohira reagent[22] (Figure S15). The Prg analogues of the inhibitors of cathepsins (leupeptin) and caspases (Ac-YVAD-CHO) showed no significant inhibitory potentials compared to the parent aldehydes (Figure S16). We postulated this was due to the low affinity of the short peptide fragments for these proteases. To test this, we synthesized two extended cyanine 5 (Cy5) fluorophore-labeled (16 and 26 amino acid) peptide fragments derived from pro-IL1-β (a natural substrate of caspase-1), carrying a C-terminal propargylated aspartic acid residue (Figure 6). Notably, both peptides selectively labeled caspase-1, and this labeling could readily be abolished by the addition of the general cysteine protease inhibitor iodoacetamide.

Caspase-1 doped into U937 cell lysate, which is low in native active caspase-1, was also selectively labeled using this probe, showing the high selectivity of the method (Figure 6). These results suggest that the reaction of C-terminal alkynes with active-site cysteine residues can be extended to other classes of enzymes.

In conclusion, we show that alkynes attached to substrate proteins and peptides can react with the active-site cysteine nucleophile of target proteases. We confirmed this for DUBs and for caspase-1. The reaction described is highly selective. The alkyne moiety does not react with excess thiol nor with cysteine residues present in nontargeted proteins, in contrast to strained cyclooctynes, which can react with cysteine nucleophiles present in serum albumin.[23] The inertness of the alkyne moiety under a

Figure 5. Quantification of DUBs precipitated from EL-4 lysate after on-bead reaction with Ub-Prg. Ratios of ion intensities of proteins retrieved from Ub-Prg-resin pulldown vs pulldown with a control resin are shown.
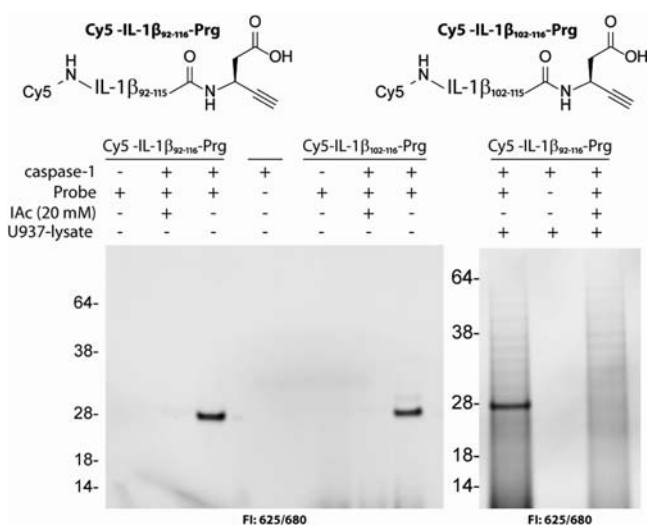
Figure 6. Labeling of recombinant caspase-1 with alkyne-based caspase-1 probe analyzed by fluorescent scanning of SDS-PAGE gel. Recombinant caspase-1 was labeled with two different lengths of caspase probe (left) and doped in U937 lysate (right), showing selective labeling of caspase-1 in lysate.

range of chemical conditions and reversibility of the linkage under acidic conditions allow direct immobilization of these probes on resin and triggered release of captured proteins. This allows for facile activity-based proteomics using very stringent purifications.

ASSOCIATED CONTENT

Supporting Information

Experimental details and characterization data. This material is available free of charge via the Internet at http://pubs.acs.org. MS proteomics data were deposited to the ProteomeXchange Consortium via the PRIDE partner repository with data set identifier PXD000073. Coordinates and structure factors for the vOTU•Ub-Prg complex have been submitted to the protein data bank, accession number 3ZNH.

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Notes
The authors declare the following competing financial interest(s): R.E., S.K., A.J., P.G., and H.O. are inventors on a Netherlands Cancer Institute patent application on the use of terminal alkynes in active-site reactive molecules. H.O. is inventor on a Netherlands Cancer Institute patent application on linear Ub total synthesis and owns shares in a company, UbiQ registered at the Netherlands Chamber of Commerce, that holds a licence to Ub synthesis technology. R.E., S.K., A.J., P.G., and H.O. are entitled to royalties resulting from technology licensing according to Netherlands Cancer Institute policies.

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