Small-Molecule Inhibitors and Probes for Ubiquitin- and Ubiquitin-Like-Specific Proteases

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The post-translational modification of proteins with ubiquitin (Ub) or ubiquitin-like (Ubl) modifiers is an important signal in the regulation of a variety of biological processes. The attachment of either a single Ub, a 76 amino acid polypeptide, or multiple Ub entities helps to control the targeting of substrates for degradation. Processes such as receptor internalization, trafficking to the lysosomal compartments, and regulation of gene expression are likewise affected by Ub conjugation.[1,2] Ubiquitin-like modifiers also play a role in protein targeting and in the regulation of protein function.[3] For instance, the small Ubl modifier SUMO, a 97 amino acid polypeptide, functions as a nuclear targeting signal, amongst other functions.[4] Nedd8, another polypeptide with homology to Ub, regulates the activity of E3 ligases, thereby influencing the ubiquitylation process.[5,6] In addition, a family of Ub related proteins, referred to as APGs, has been shown to control autophagy.[7,8]

The reversibility of protein modification with Ub/Ubl resembles a phosphorylation/dephosphorylation cycle, and is essential to allowing control over this modification. At least 400 specific Ub ligases, responsible for the attachment of Ub to protein substrates, are thought to exist.[9,10] The reverse reaction, removal of Ub moieties from substrates, is performed by the large family of Ub-specific proteases, USPs (also referred to as deubiquitinating enzymes, DUBs).[11–13] The attachment and removal of Ubl modifiers is carried out by a less numerous set of enzymes.[14,15]

Insight into the enzymatic function of individual DUBs has been obtained for several members, including USP7,[16,17] USP8,[18,19] UCH37,[20] USP14,[21,22] IsoT,[23,24] USP9,[25] BAP1,[26] UCH-L1,[27,28] and UCH-L3.[29] However, the physiological role of many proteases of this enzyme class remains largely uncharacterized. In addition, a high degree of functional redundancy, as inferred from mutation analysis in yeast, complicates the study of the biological roles for individual USPs (Casagrande and Ploegh, unpublished data). Many Ubls have a single conjugation system and only a few deconjugating enzymes have been identified to date for SUMO, Nedd8, ISG15, and APG8.[15]

An attractive strategy to further our understanding of proteolytic enzymes is the design of selective inhibitors. Indeed, this approach has been successfully applied to the caspase[30] and cathepsin[31] families as well as the individual enzymatic activities of the proteasome.[32] Despite considerable research efforts in recent years, there is still a lack of selective small-molecule synthetic inhibitors that target USPs and Ubl-specific proteases.

The first generation of USP and Ubl-specific protease inhibitors is based on the entire Ub/Ubl protein itself modified at the C terminus with electrophilic entities capable of reacting with the active-site cysteine thiol, present in most Ub and Ubl-specific proteases. These electrophilic traps include aldehydes (Ubal),[33,34] nitrile derivatives,[20] Michael acceptors (including vinyl sulfone, vinyl methyl ester), and alkyl halides.[35] Different C-terminally modified Ub moieties allow the profiling of enzyme activity, and show the selectivity of these probes for subsets of the corresponding proteases.[35] To date, only a few examples of small molecules with inhibitory potential toward USPs have been reported.[36–38] However, these examples exhibit only moderate activity and selectivity. Unlike other proteolytic enzymes, USPs require a considerable portion of the Ub moiety—in addition to the electrophilic trap mimicking the isopeptide linkage—for optimal recognition.[39,40] Based on the structural similarity between Ub and Ubl proteins, this most likely holds true also for Ubl-specific proteases,.[41]

Here we report the synthesis of a panel of peptide vinyl sulfones harboring various portions of the Ub C terminus. We show that this strategy can be applied also to the synthesis of C-terminal peptide vinyl sulfones corresponding to the Ubl modifiers Nedd8, SUMO 1, FAT10, Fau, and APG12. Depending on their length, such compounds can efficiently target USPs and Ubl-specific proteases.

Our synthetic strategy toward C-terminally modified Ub/Ubl peptides is based on the use of Kenner's safety-catch linker, recently revitalized by Ellman and co-workers[42] and applied by us for the synthesis of peptide vinyl sulfone proteasome inhibitors.[43,44] Briefly, standard Fmoc-based solid-phase peptide synthesis starting from sulfonamide resin **1** afforded immobilized oligopeptide **2**, the N terminus of which is equipped with a biotin moiety, and **5**, with a N-terminal, benzyl oxycarbonyl (Z)

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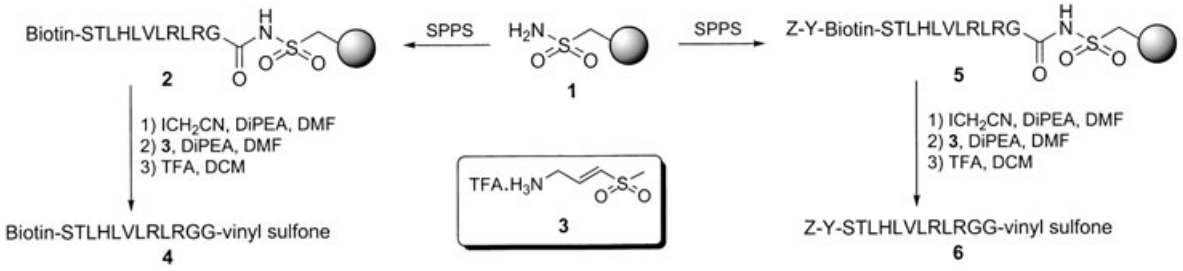

Scheme 1. *Synthesis of truncated ubiquitin C-terminal vinyl sulfone-based probes by using a safety-catch approach.*

protected tyrosine residue for ensuing radioiodination (Scheme 1, the side-chain protective groups in **2** and **5** are omitted for clarity). After activation of the acylsulfonamide resin by alkylation with iodoacetonitrile, cleavage from the resin with concomitant installation of the C-terminal vinyl sulfone was realized by treatment with glycine vinyl sulfone derivative **3** and diisopropylethylamine (DiPEA). Finally, the acid-labile side-chain protective groups were removed by treatment with TFA, to provide the target peptide vinyl sulfones **4** and **6**. In this fashion, a panel of 5-mer to 18-mer peptide vinyl sulfones, corresponding to the C-terminal sequence of ubiquitin, was prepared (Table 1). Yields after HPLC purification were typically in the range of 10–15% with a high purity (for a representative example of HPLC purification and MS analysis, see Figure 1), and mg quantities were obtained in all cases. By using the same strategy, N-terminally biotinylated oligomers corresponding to the C terminus of the Ubl modifiers Nedd8 (**15**), SUMO1 (**16**–**18**), APG12 (**19**–**21**), FAT10 (**22**), and Fau (**23**) were prepared with comparable efficiency and purity. Increased yields in biotin incorporation were achieved when a biotin–aminohexanoic acid spacer was used (biotin-Ahx_1, for **16**–**23**). The compounds **20**–**22** contain a cysteine residue that might react with the C-terminal vinyl sulfone moiety. However, we did not observe evidence of any cyclization or dimerization reactions, since **20** and **21** were able to modify polypeptide species (see Figure 4A, lanes 3, 4, below).

The sequences are from the mouse proteins with the following accession numbers (http://www.ncbi.nlm.nih.gov): ubiquitin: P62991; Nedd8: AAH04625; SUMO1: NP_033486; APG12: BAB62092; FAT10: P63072; Fau: I483346. The corresponding C-terminal sequences are identical in mouse and man with the exception of FAT10.

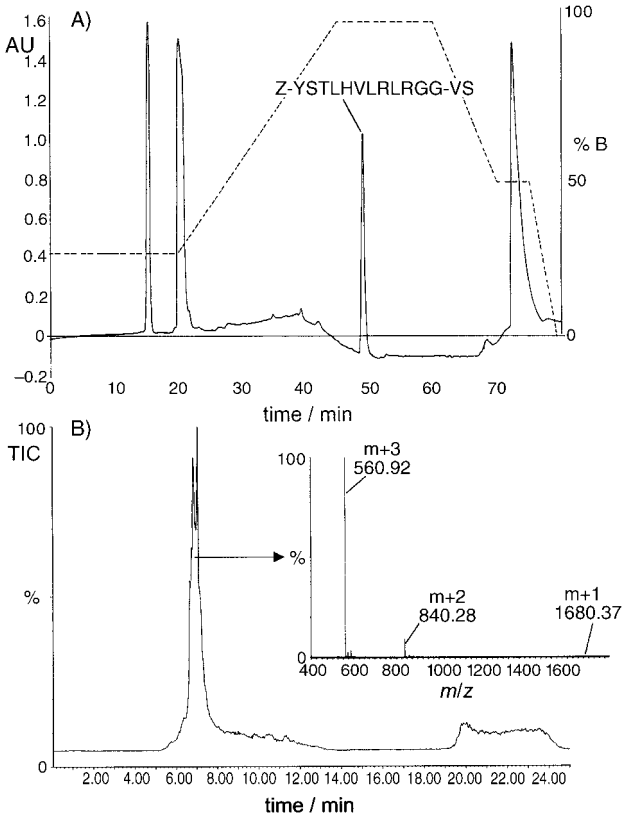

Figure 1. *HPLC purification and MS analysis of peptide vinyl sulfone* ***6****. A) UV (214 nm) HPLC analytical chromatogram of compound Z-YSTLHVLRLRGG-vinyl sulfone (****6****). B) LC-MS analysis of compound* ***6*** *performed on a single quadrupole mass spectrometer (Waters, MA, USA) coupled to a HPLC system (Hewlett–Packard HP1100) reveals the product with a mass of* $M_r = 1680.37$ *[M+H]*$^+$*. The theoretical mass is* $M_r = 1679.02$.

Table 1. *List of the truncated ubiquitin and ubiquitin-like modifier probes prepared in this study.*

Ub peptide vinyl sulfones	Ubl peptide vinyl sulfones
Z-RLRGG-VS (**7**)	Biotin-SVLHLVLALRGG-VS (**15**) (Nedd8)
Z-LRLRGG-VS (**8**)	Biotin-Ahx_1EQTGG-VS (**16**) (SUMO1)
Z-LVLRLRGG-VS (**9**)	Biotin-Ahx_1EVYQEQTGG-VS (**17**) (SUMO1)
Z-LHLVLRLRGG-VS (**10**)	Biotin-Ahx_1EDVIEVYQEQTGG-VS (**18**) (SUMO1)
Z-STLHLVLRLRGG-VS (**11**)	Biotin-Ahx_1SQAWG-VS (**19**) (APG12)
Biotin-STLHLVLRLRGG-VS (**4**)	Biotin-Ahx_1HYCKSQAWG-VS (**20**) (APG12)
Z-Y-STLHLVLRLRGG-VS (**6**)	Biotin-Ahx_1LVLHYCKSQAWG-VS (**21**) (APG12)
Z- KESTLHLVLRLRGG-VS (**12**)	Biotin-Ahx_1SLLFLTTHCTGG-VS (**22**)(FAT10)
Z-IQKESTLHLVLRLRGG-VS (**13**)	Biotin-Ahx_1TTLEVAGRMLGG-VS (**23**) (Fau)
Z-YNIQKESTLHLVLRLRGG-VS (**14**)	

As the next research objective, we set out to establish the inhibitory activity of the peptide vinyl sulfone series **7**–**14**. Crude lysates prepared from the EL-4 mouse thymoma cells were incubated with **7**–**11** at 1, 50, and 100 μM for 2 h prior to treatment with [^{125}I]-UbVS, a broad-spectrum USP-specific probe previously reported from our laboratory.[21] As can be seen in Figure 2, labeling of a variety of polypeptides corresponding to active USPs present in crude extracts can be effectively abolished by 12-mer **11**, but not by the smaller pep-

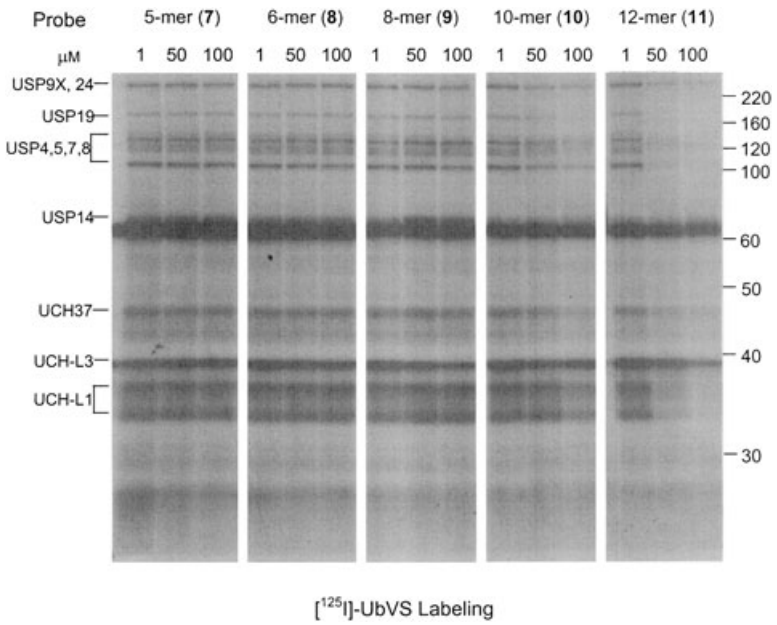

Figure 2. *Optimal-length requirements for targeting USPs by truncated ubiquitin probes. Crude extracts were prepared from mouse thymoma cells (EL-4), and cell lysate (50 μg per sample) was incubated with the indicated concentrations of the 5-mer (**7**), 6-mer (**8**), 8-mer (**9**), 10-mer (**10**), or the 12-mer (**11**) ubiquitin-based probe for 30 min at 37°C. [125I]-Ub-VS, prepared as described,[21] was then added, followed by incubation for 1 h at 37°C. Labeled crude extracts were separated by SDS-PAGE (10%) and analyzed by autoradiography. Targeted USPs are indicated based on a functional proteomics study as described previously.[35]*

tide vinyl sulfones (**7**–**10**). The observation that extended C-terminal peptides are required for effective USP inhibition is underscored by the finding that the commercially available fluorogenic pentapeptide substrate RLRGG-AMC, with amino an acid sequence that corresponds to peptide vinyl sulfone **7**, can be used to identify USP activity only at elevated concentrations. The available structural data also point in the direction of a requirement for extended C-terminal peptide fragments to achieve efficient USP binding. Inhibition of labeling did not further improve when using 14-mer **12**, 16-mer **13**, or 18-mer **14** as competitors (data not shown). We conclude that, at least from this series, the truncated peptide vinyl sulfone corresponding to the 12 C-terminal amino acid residues is the most effective probe.

Next, we tested whether 12-mer peptide vinyl sulfones are able to target a recombinant, purified DUB, the Ub C-terminal hydrolase UCH-L3. For this purpose, we expressed and purified UCH-L3 enzyme.[45] UCH-L3 was incubated with decreasing amounts of biotinylated Ub 12-mer **4** (Figure 3A, lanes 2–5). The adducts were then analyzed by SDS-PAGE, followed by streptavidin-HRP detection. The bands in lanes 3–5 correspond to the covalent complex of UCH-L3 and the biotinylated 12-mer. The addition of the generic S-alkylating agent *N*-ethylmaleimide (NEM, lane 2) effectively abolishes labeling; this indicates that UCH-L3 modification occurs through Michael reaction of the active site thiol with the vinyl sulfone moiety in **4**. In a similar fashion, radioiodinated peptide vinyl sulfone **6** proved to be effective in labeling UCH-L3, as visualized in the autoradiogram in Figure 3B. (Introduction of [125I] in **6** was accomplished following a previously established protocol.[44]) As seen in panel C, incubation with 12-mer **4** leads to the appearance of a new band corresponding to the UCH-L3–12-mer adduct. The appearance of a single band is consistent with the suggestion that only the active-site cysteine of UCH-L3 is modified by the vinyl sulfone.

We extended our approach, and prepared 12-/13-mer probes corresponding to the C termini of Nedd8, SUMO1, APG12, FAT10, and Fau (**15, 18, 21**–**23**). Initial labeling experiments of crude EL-4 extracts indicated that the SUMO1 13-mer and APG12 12-mer probes targeted specific polypeptides. These compounds were therefore examined in further detail. The labeling of EL-4 lysates with the selected Ubl probes is presented in Figure 4. The lysates were incubated with the C-terminal truncated 5-mer, 9-mer, and 12- or 13-mer at a concentration of 2 μM for 15 min for the APG12 peptides and at a 5 μM concentration for 1 h for the SUMO1 peptides, respectively. Whereas the APG12 5-mer **19** does not seem to modify any target at the concentrations applied (the two visible bands correspond to nonspecific labeling of background material normally observed in biotin–streptavidin blots), addition of the corresponding 9- and 12-mers **20** and **21** to EL-4 lysate resulted in the labeling of several polypeptides in the 47 kDa range (Figure 4A). To our surprise, the set of SUMO1 probes **16**–**18**

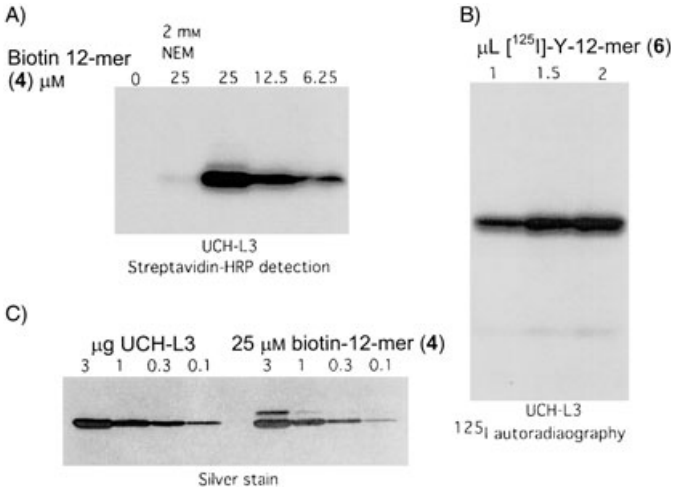

Figure 3. *Specific labeling of UCH-L3 with ubiquitin C-terminal 12-mer probe **4**. A) Labeling of UCH-L3 (1 μg per lane) with **4** in a dose-dependent manner. B) Labeling of UCH-L3 (1 μg per lane) with [125I] radiolabeled **6**. C) Labeling of UCH-L3 with **4** reveals a 1 kDa shift in molecular weight, corresponding to the covalent enzyme–probe adduct.*

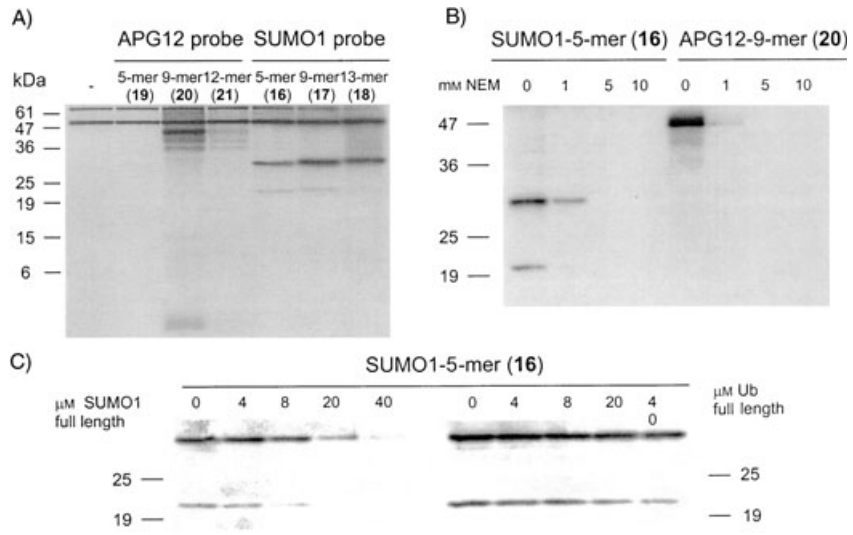

Figure 4. *Probing for Ubl-specific hydrolases requires shorter C-terminal peptide fragments than for USPs. A) Labeling of EL-4 lysate with APG12 C-terminal 5-mer, 9-mer, and 12-mer (**19**–**21**) and SUMO1 C-terminal 4-mer, 8-mer, and 12-mer probes (**16**–**18**). B) Labeling of EL-4 lysate with **16** and **20** can be abolished by the addition of the S-alkylating agent N-methylmaleimide. C) Competition of SUMO1 5-mer **16** labeling of EL-4 lysate with full-length SUMO1 and full-length ubiquitin protein.*

appeared to be equally efficient at labeling a specific set of proteins in EL-4 lysate at 20 and 30 kDa. It appears that, in this case, 5 amino acid residues are sufficient to achieve selective targeting. With both the SUMO1 and APG12 probes, addition of N-ethyl maleimide resulted in the disappearance of the labeled proteins (Figure 4B). This showed that all of the labeled proteins were modified through the interaction with the vinyl sulfone moiety of the Ubl probes and thus contain an active-site thiol nucleophile. Furthermore, competition experiments demonstrated that labeling with the SUMO1 5-mer probe is specific for a SUMO1 protease (Figure 4C). To illustrate specificity, EL-4 lysates were incubated with either full-length SUMO1 or full-length Ub protein prior to the addition of the electrophilic SUMO1 5-mer. An increasing concentration of full-length SUMO1 abolished the labeling with the 5-mer, while the addition of full-length Ub did not have any effect on the labeling pattern. Likewise, we observed that labeling with the APG12 9-mer **20** was not inhibited by an excess of full-length ubiquitin. Competition experiments with intact APG12 were not performed because of difficulties in expression and isolation of the pure protein.[15] Consistent with these results, the SUMO 5-mer and APG12 9-mer probes failed to label recombinant UCH-L3; this further indicated the specificity and selectivity contained in the C-terminal region Ubls (data not shown).

In conclusion, we have demonstrated the efficacy of Kenner's safety-catch linker in the preparation of extended peptide vinyl sulfones corresponding to the C terminus of Ub/Ubl. Depending on the number of residues incorporated, these C-terminally modified peptides proved capable of modifying recombinant UCH-L3 and were effective in competing with full-length Ub-VS in DUB inhibition in complex biological mixtures. Preliminary studies indicate that truncated Ubl peptide vinyl sulfones are capable of modifying Ubl-specific proteases in a manner similar to that of the Ub-based peptide vinyl sulfones. Although only two Ubl proteins were examined in further detail, we observed that specificity is retained in a shorter C-terminal stretch for Ubl-specific proteases as compared to USPs. The reason for this discrepancy is not clear. However, the structure of Ub aldehyde–yuh1, Ub–UBP7, and SUMO aldehyde–ULP1 complexes showed that the mode of Ub recognition is substantially different from that of SUMO. ULP1 and UBP7 both recognize the C-terminal Gly–Gly motif but by using distinct modes of binding. Also, a smaller surface area of SUMO is recognized by its cognate enzyme; this might indicate that specificity is confined to a smaller molecular domain on the substrate. This is consistent with the observation that only a small number of proteases recognize Ubl proteins, whereas many different DUBs, spanning several enzyme families, can recognize the ubiquitin domain. The Ub-recognition motif appears to be more universal and used in the regulation of a large variety of different biological processes.

Current research is focused on establishing the DUB specificity of the developed probes and on the application of tandem mass spectrometry-based protocols for the identification of the modified proteins targeted by the truncated Ub/Ubl peptide vinyl sulfones.

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