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Designed Semisynthetic Protein Inhibitors of Ub/Ubl E1 Activating Enzymes

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Ubiquitin (Ub) and related ubiquitin-like (Ubl) proteins such as SUMO serve as reversible, post-translational modifications of protein substrates, impacting diverse cellular processes.[1,2] The Ub/Ubl modifier is coupled by its C-terminal carboxylate to specific lysine side chains on target proteins via an isopeptide bond. Initial steps in this process are catalyzed by a Ub/Ubl activating enzyme (E1), which first adenylates the Ub/Ubl C-terminus to form a Ub/Ubl-AMP intermediate and then transfers the Ub/Ubl to a conserved cysteine on the E1 (Figure 1). The Ub/Ubl is then transthioesterified onto a cysteine side chain of a conjugating enzyme (E2) and finally transferred to a lysine side chain of the target protein, often mediated by a ligase (E3). Although structures of several E1s have been reported,[3,4] outstanding questions remain about the mechanisms of these reactions. First, E1s surprisingly crystallize with substrates bound in the active site rather than the in situ-formed, presumably more tightly binding acyl-AMP intermediate,[5] in contrast to other enyzmes that catalyze adenylation reactions.[6] Second, the conserved E1 cysteine that serves as the nucleophile in the thioesterification half-reaction is remote, >30 Å away from the adenylation active site. These observations suggest that additional conformational changes are required in both half-reactions.[3] To investigate these questions, we sought to develop mechanism-based inhibitors of E1s that could then be used in pivotal structural and biochemical studies.[7] Selective inhibitors would also be useful probes for dissecting E1 functions. We and others have used 5′-sulfonyladenosine-based small molecules to inhibit various mechanistically (but not structurally) related enzymes that catalyze adenylation reactions.[8] We envisioned that such inhibitor design strategies might also be effective for E1s and report herein the development of semisynthetic, C-terminally modified Ub/Ubl proteins as novel, selective E1 inhibitors.

Our initial efforts focused on small-molecule inhibitors consisting of the conserved C-terminal diglycine motif of Ub/Ubl modifiers linked covalently to a 5′-*O*-sulfamoyladenosine (AMS) nonhydrolyzable analogue of AMP (Figure S1 in the Supporting Information).[9] However, these compounds proved to be extremely weak E1 inhibitors.[9] Thus, to develop more potent inhibitors, we investigated 5′-sulfonyladenosine-based modifications to the C-termini of full-length Ub/Ubl proteins.

Examination of the Ub/Ubl·E1 cocrystal structures revealed that the conserved C-terminal diglycine motif is preceded by a non-conserved hydrophilic residue with a solvent-exposed side chain not bound to the E1 or Ub/Ubl.[3] This suggested that this residue could be replaced with a cysteine to enable native chemical ligation of synthetic tripeptides having C-terminal 5′-sulfonyladenosine-based modifications and an N-terminal cysteine to truncated Ub/Ubl$^{\Delta}$ proteins having a C-terminal thioester (Figure 2).[10] Thus, we

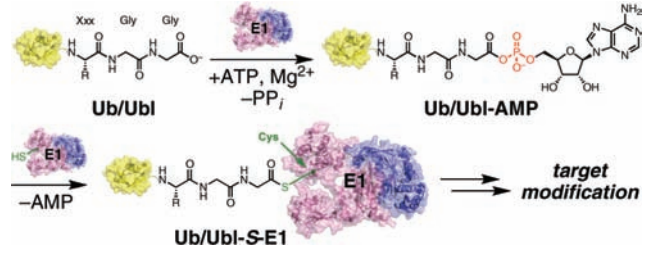

Figure 1. Ub/Ubl activating enzymes (E1) catalyze adenylation of a Ub/Ubl at its C-terminus followed by thioesterification at a conserved cysteine of the E1 (Uba2 Cys173 in SUMO E1; Uba1 Cys593 in Ub E1).[2]

synthesized tripeptides CGG-AMSN (**1**) and CGG-AVSN (**2**) and ligated them to SUMO$^{\Delta}$ (**3a**) and Ub$^{\Delta}$ (**3b**) thioesters produced by the intein fusion protein method.[9,11] SUMO-AMSN (**4a**) and Ub-AMSN (**4b**) contain a sulfamide as a nonhydrolyzable analogue of the phosphate in the Ub/Ubl-AMP intermediate in order to probe the first half-reaction. SUMO-AVSN (**5a**) and Ub-AVSN (**5b**) contain a vinyl sulfonamide electrophile designed to trap the incoming cysteine nucleophile in the second half-reaction.[12]

We then set out to test the abilities of these constructs to bind and inhibit SUMO E1 (Sae1·Uba2) and Ub E1 (Uba1). Gel-filtration experiments demonstrated that SUMO-AMSN (**4a**) binds SUMO E1 (Figure S2).[9] Moreover, **4a** effectively inhibits Uba2-*S*-SUMO thioester formation (Figure 3a) as well as subsequent E1-dependent SUMO conjugation to a substrate protein, RanGAP (Figure S3), both in a dose-dependent fashion that can be overcome with excess SUMO. This inhibition presumably occurs at the level of the first half-reaction. Ub-AMSN (**4b**) similarly inhibits Uba1-*S*-Ub thioester formation in a dose-dependent manner (Figure 3b). Importantly, these two inhibitors are highly selective for their cognate E1s and do not inhibit the corresponding noncognate E1s (Figure 3c).

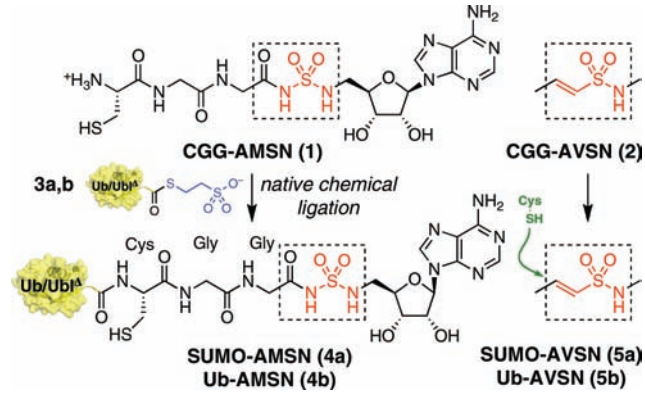

Figure 2. Semisynthesis of mechanism-based SUMO E1 and Ub E1 inhibitors **4** and **5** (Ub/Ubl$^{\Delta}$ = SUMO$^{1-94}$; Ub$^{1-71}$).[2]

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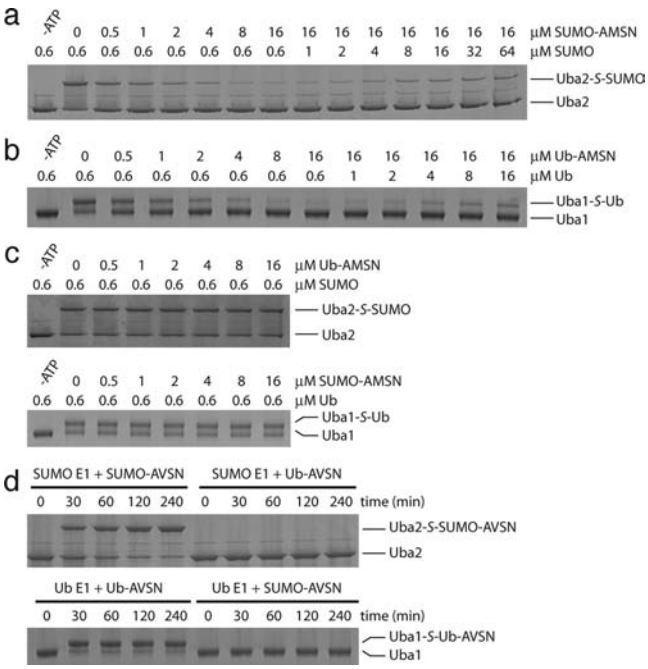

Figure 3. Inhibition of SUMO E1 (Sae1·Uba2) and Ub E1 (Uba1) by semisynthetic, C-terminally modified SUMO and Ub constructs (SDS−PAGE data).[9] (a, b) SUMO-AMSN (**4a**) inhibits SUMO E1-*S*-SUMO thioester formation, and Ub-AMSN (**4b**) inhibits Ub E1-*S*-Ub formation in a dose-dependent manner. (C) The constructs do not inhibit the noncognate E1s. (d) SUMO-AVSN (**5a**) covalently cross-links to SUMO E1 (Uba2 subunit) but not to Ub E1 (Uba1), and Ub-AVSN (**5b**) covalently cross-links to Ub E1 (Uba1) but not to SUMO E1 (Uba2 subunit).

We next investigated the ability of SUMO-AVSN (**5a**) to cross-link covalently to the Uba2 subunit of SUMO E1, which contains the nucleophilic Cys173. As hoped, incubation of SUMO-AVSN (**5a**) with SUMO E1 led to the formation of a putative Uba2-*S*-SUMO-AVSN thioether adduct with a concomitant decrease in the level of native Uba2 (Figure 3d). Cross-linking did not compromise the ability of the Uba2 subunit to complex with the Sae1 subunit (Figure S4)[9] and was not observed when SUMO-AVSN (**5a**) was incubated with a mutant SUMO E1 lacking the cysteine nucleophile, Uba2 C173S (Figure S5).[9] Furthermore, the thioether adduct was stable to thiolysis by dithiothreitol, in contrast to the native Uba2-*S*-SUMO thioester product (Figure S6).[9] Finally, the preformed adduct was unable to promote SUMO conjugation to RanGAP (Figure S7).[9] Ub-AVSN (**5b**) similarly cross-linked Ub E1 (Uba1) (Figure 3d) but not a mutant lacking the cysteine nucleophile, Uba1 C593A (Figure S8).[9] These two inhibitors were again selective for their cognate E1s and did not cross-link to the corresponding noncognate E1s (Figure 3d). Taken together, these data demonstrate that SUMO-AVSN (**5a**) and Ub-AVSN (**5b**) form the desired E1-*S*-Ub/Ubl-AVSN adducts via stable thioether linkages to the conserved nucleophilic cysteine, thus halting the Ub/Ubl conjugation process at the level of the second half-reaction.

In conclusion, we have developed mechanism-based, semisynthetic protein inhibitors of the SUMO E1 and Ub E1 activating enzymes. In structural and biochemical studies reported elsewhere,[13] these inhibitors have provided striking new insights into the mechanisms of E1-catalyzed adenylation and thioesterification. Furthermore, these inhibitors are highly selective for their cognate E1 enzymes, highlighting the utility of designed protein substrate analogues in achieving inhibitor selectivity,[14] and can be used in the future to dissect the biological functions of E1 enzymes.

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Supporting Information Available: Complete ref 7, detailed procedures, and analytical data for all new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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