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Designed Semisynthetic Protein Inhibitors of Ub/Ubl E1 Activating Enzymes

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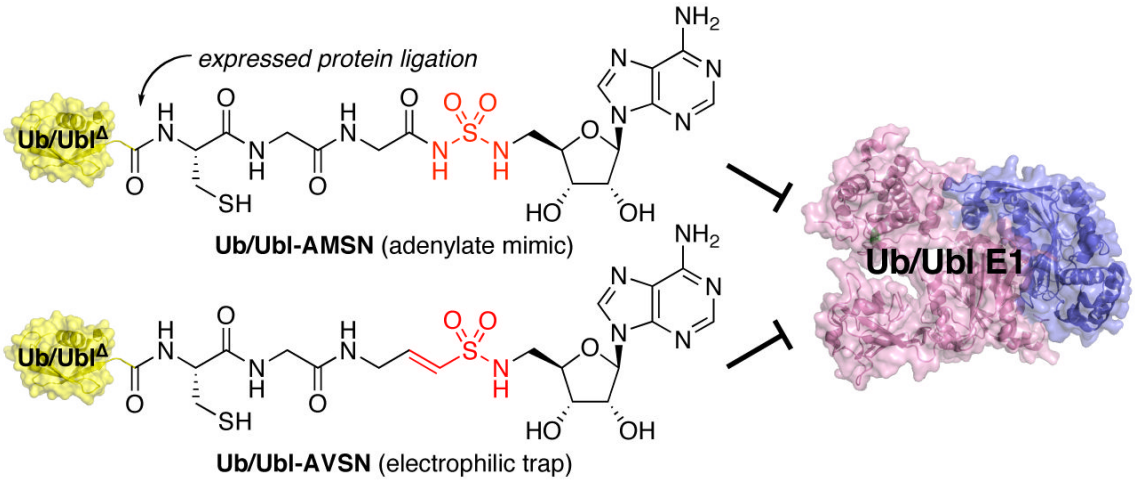
Abstract



Semisynthetic, mechanism-based protein inhibitors of ubiquitin (Ub) and ubiquitin-like modifier (Ubl) activating enzymes (E1s) have been developed to target E1-catalyzed adenylation and thioesterification of the Ub/Ubl C-terminus during the processes of protein SUMOylation and ubiquitination. The inhibitors were generated by intein-mediated expressed protein ligation, using a truncated Ub/Ubl protein (SUMO residues 1–94; Ub residues 1–71) with a C-terminal thioester, and synthetic tripeptides having a C-terminal adenosine analogue and an N-terminal cysteine residue. SUMO-AMSN (**4a**) and Ub-AMSN (**4b**) contain a sulfamide group as a non-hydrolyzable mimic of the phosphate group in the cognate Ub/Ubl-AMP adenylate intermediate in the first half-reaction, and these constructs selectively inhibit SUMO E1 and Ub E1, respectively, in a dose-dependent manner. SUMO-AVSN (**5a**) and Ub-AVSN (**5b**) contain an electrophilic vinyl sulfonamide designed to trap the incoming E1 cysteine nucleophile (Uba2 Cys173 in SUMO E1; Uba1 Cys593 in Ub E1) in the second half-reaction, and these constructs selectively, covalently, and stably crosslink to SUMO E1 and Ub E1, respectively, in a cysteine nucleophile-dependent manner. These inhibitors

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are powerful tools to probe outstanding mechanistic questions in E1 function and can also be used to study the biological functions of E1 enzymes.

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 sidechains 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, 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 tighter 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 comprised of the conserved C-terminal diglycine motif of Ub/Ubl modifiers, linked covalently to a 5′-*O*-sulfamoyl-adenosine (AMS) non-hydrolyzable analog of AMP (Figure S1).[9] However, these compounds proved extremely weak E1 inhibitors.[9] Thus, to develop more potent inhibitors, we investigated 5′-sulfonyladenosine-based modifications to the C-terminus 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 sidechain 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 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 non-hydrolyzable analog of the phosphate in the Ub/Ubl-AMP intermediate 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, SUMO-AMSN (**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 could be overcome with excess SUMO. This inhibition presumably occurs at the level of the first half-reaction. Ub-AMSN (**4b**) similarly inhibited Uba1-*S*-Ub thioester formation in a dose-

dependent manner (Figure 3b). Importantly, these two inhibitors were highly selective for their cognate E1s and did not inhibit the corresponding non-cognate E1s (Figure 3c).

We next investigated the ability of SUMO-AVSN (**5a**) to crosslink 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 formation of a putative Uba2-*S*-SUMO-AVSN thioether adduct, with a concomitant decrease in the level of native Uba2 (Figure 3d). Crosslinking 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] Further, the thioether adduct was stable to thiolysis by DTT, 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 crosslink to the corresponding non-cognate 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 in the accompanying paper, these inhibitors have provided striking new insights into the mechanisms of E1-catalyzed adenylation and thioesterification.[13] Further, 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 to dissect the biological functions of E1 enzymes in the future.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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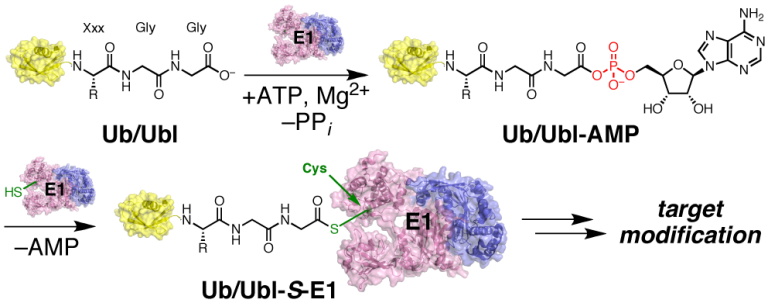

Figure 1.

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]

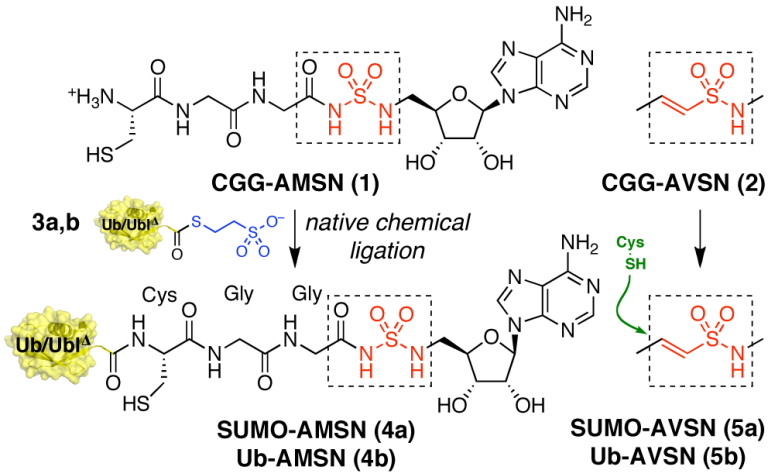

Figure 2.
Semisynthesis of mechanism-based SUMO E1 and Ub E1 inhibitors (**4**, **5**). (Ub/Ubl$^{\Delta}$ = SUMO$^{1–94}$; Ub$^{1–71}$).[2]

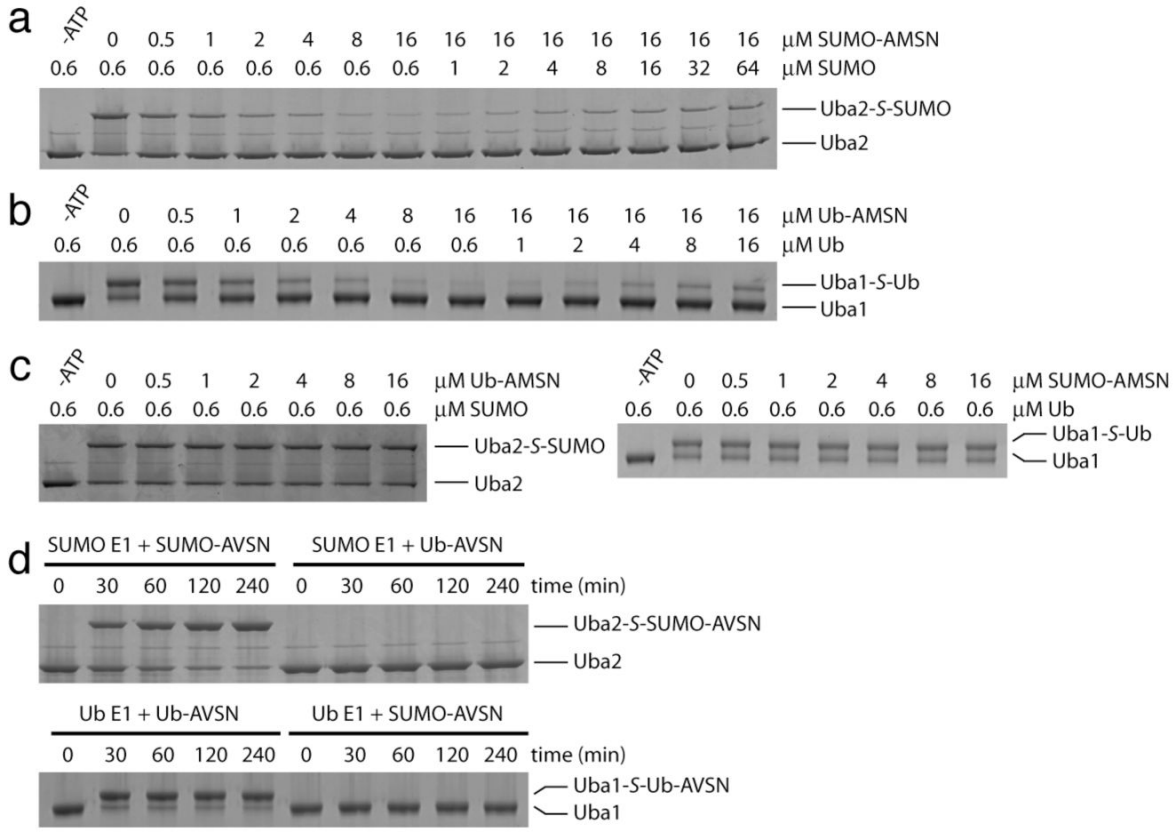

Figure 3.

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 non-cognate E1s. (d) SUMO-AVSN (**5a**) covalently crosslinks to SUMO E1 (Uba2 subunit) but not to Ub E1 (Uba1) and Ub-AVSN (**5b**) covalent crosslinks to Ub E1 (Uba1) but not to SUMO E1 (Uba2 subunit).