A Novel Inhibitor of SUMOylation Pathway: Understanding the Mechanism of Action

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ABSTRACT
SUMOylation, one of the many important post-translational modifications in eukaryotes, is a process in which small ubiquitin-like modifiers (SUMO) is covalently attached to other protein substrates and regulates various cellular activities in vivo. Extensive studies have shown that dysregulations of SUMOylation are involved in various human diseases, such as neurodegenerative diseases and numerous types of cancers. Currently, no SUMOylation inhibitor has been found. From our high throughput screening for SUMOylation inhibitor discovery, a compound STE was found to inhibit SUMOylation and preferentially kill lung cancer cells. In this paper, the specific step that is inhibited by STE was explored through determining E1~SUMO and E2~SUMO conjugates by gel-based assays. STE appears to affect the E1~SUMO thioester bond formation and this result will provide an important tool to understanding SUMO’s role in physiology and disease. Furthermore, it will facilitate drug discovery for cancer treatments and therapeutics.

Keywords: SUMOylation, small ubiquitin-like modifier, small molecule inhibitor, high throughput screening, novel cancer therapy, E1 activating enzyme, E2 conjugating enzyme, and E3 ligase.

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Professor Liao’s research includes studies on signal transduction pathways, small ubiquitin-like modifier ligase, G protein-coupled receptors, and lipid receptors. He has developed several high-throughput screening systems for drug candidates. He has found evidence recently for several fatty acid receptors that may play an important role in understanding fatty acid induced insulin resistance and the relationship between obesity and diabetes.
INTRODUCTION

Small ubiquitin-like modifiers (SUMO) are covalently attached to certain residues of protein substrates, therein modifying substrate activities as a post-translational modification. Posttranslational modification by SUMO affects as well as regulates diverse cellular processes such as nuclear transport, transcriptional regulation, cell progression, signal transduction, and cell cycle progression [1]. SUMOylation is a dynamic process in which SUMO can undergo the enzymatic cascade composed of E1, E2, and E3 ligases to conjugate to specific protein substrates, but SUMO can be cleaved from protein substrates through SENPs (sentrin-specific proteases) as shown in Figure 1 [2]. Thus, this free SUMO can be used to undergo conjugation to other protein substrates [3].

SUMOylation Conjugation

The SUMOylation pathway (Fig. 2) is catalyzed by an enzymatic cascade that involves three enzymes: E1 (Aos1/Uba2), E2 (Ubc9), E3 ligases such as PIAS [4]. First, SUMO is matured from the SUMO precursor form through removing the C-terminal peptide by SENPs [5]. Second, the SUMO-activating enzyme E1, a heterodimer composed of Aos1/Uba2, undergoes an ATP-dependent step to adenylate the C-terminal carboxyl group of SUMO, therein releasing pyrophosphate [6]. Next, SUMO is transferred to the thiol group of the catalytic cysteine of E1, releasing AMP and Aos1 and forming a high-energy thioester bond between E1 (Uba2) and the C-terminus of SUMO (E1–SUMO) [5]. SUMO is then transferred to a cysteine of the SUMO-conjugating enzyme E2, Ubc9, forming another high-energy thioester bond (E2–SUMO) [6]. Lastly, SUMO is transferred from E2 and conjugated to a substrate at the Lys residue with the assistance of SUMO ligases – E3 in vivo [6].

SUMO and Cancer

The SUMOylation pathway is a potential drug target for cancer treatment because extensive studies have shown that the upregulation of SUMOylation, meaning the over-expression of SUMO components, are shown in numerous cancers such as breast cancer, lung adenocarcinomas, ovarian tumors, and Myc-driven tumorigenesis [1, 7]. For example: Ubc9 is overexpressed in tumors, such as lung and ovarian carcinomas [1]. Moreover, research has shown that SUMOylation plays an integral role in the critical signaling pathways of tumorigenesis. For example, Uba2 (E1 enzyme) is required for the growth of Myc-overexpressed breast cancer [7]. SUMOylation is necessary for tumor formation in that the SAE2 (E1) inactivation causes mitotic catastrophe that leads to cell death Myc hyperactivation [7]. Therefore, we hypothesized that if SUMOylation is targeted and inhibited, then it may decrease cancer cell viability.

Here, we report the characterization of STE as an inhibitor of the SUMOylation pathway and specifically as an inhibitor of the E1 thioester reaction. This compound was found through FRET-based in vitro HTS assay in our lab[2,8]. In preliminary studies, STE has been discovered to preferentially kill cancer cells [9,10]. We characterize
STE as an inhibitor of the SUMOylation pathway by determining E1–SUMO and E2–SUMO conjugates through gel-based assays and analyzing the effect of STE on the E1–SUMO and E2–SUMO thioester bond formations.

**Methods**

**FRET-based High Throughput Screening**

Currently, no specific and potent SUMOylation inhibitor has been found. However, through the method of FRET-based high throughput screening, Dr. Liao’s laboratory screened approximately 220,000 compounds in an effort to identify SUMOylation inhibitors, as published previously by our group [2,8]. Förster Resonance Energy Transfer (FRET) is a technique in which proteins are tagged with the CFP-YFP fluorescence pair that is excited at 414 nm to observe protein-protein interactions as shown in Figure 3 [2, 10]. When CFP is excited at 414 nm it will emit energy around 475 nm that can excite the YFP when

![Fig. 3 FRET-based high throughput screening method utilized to identify a small molecule inhibitor for the SUMOylation pathway. The two proteins are close to each other (1-10 nm). The YFP excitation will emit energy at 530 nm. However, if there is no interaction between the tagged proteins, then it should only result in one emission peak at 475 nm when excited at 414 nm [2].](image)

In the screening to discover inhibitors for SUMOylation, 220,000 molecules and CFP-SUMO were aliquoted in 384 well plates and excited at 414 nm; the emission of 530 nm was measured first and this will serve as a control – ensuring there is no signal interference from the compound itself - to classify whether a compound has inhibited the SUMOylation pathway. A second measurement was taken after adding the E1 (Aos1/Uba2), E2 (Ubc9), and the substrate (YFP-RanGAP1) at the excitation of 414 nm. The E3 ligase was not necessary in in vitro SUMOylation of RanGAP; therefore we did not add any E3 ligase in this screening. The emission ratios of all the wells were calculated and compared to look for compounds that decreased the emission ratio [2].

**Determination of E~SUMO and E2~SUMO Thioester Conjugates by Gel-based Assay**

To determine which step STE inhibits in SUMOylation, we used a gel-based assay to determine E1–SUMO and E2–SUMO conjugates. To perform both E1–SUMO and E2–SUMO thioester assays, we used proteins that were expressed in the E. coli system. The E1–SUMO thioester formations were conducted in SUMOylation buffer (50 mM Tris [pH 7.5], 5 mM MgCl2, 0.1 mM DTT). Different concentrations of STE ranging from 0.1-25 μM or DMSO were combined with 3 μM SUMO and 1 μM E1(Aos1/Uba2). The E1–SUMO thioester reactions were started with the addition of 2 mM ATP and were incubated in 37°C water bath for 10 min to allow the SUMOylation reaction to occur with seven different concentrations of STE (0.1 μM, 0.5 μM, 1 μM, 3 μM, 5 μM, 10 μM, and 25 μM) as shown in Figure 4 [11]. The reactions were terminated by adding non-reducing 4 M urea/SDS-gel loading buffer. At the end of the assay, an additional aliquot was taken and mixed with reducing SDS-loading buffer containing DTT and heated to 95°C for 5 min. DTT is a strong reducing agent that reduces the disulfide bonds of proteins including thioester bonds; thus, it was used as a negative control for the thioester bond formation. Lastly, the protein samples were then subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) [11,12].

To visualize the protein bands corresponding to the thioester intermediate formation, the SDS PAGE gel was analyzed through Coomassie Blue staining and western blotting. Coomassie Blue staining is a technique used to visualize proteins in the gel by comparing the band on the
gel with the MW on the ladder [13]. However, Coomassie Blue staining is not specific to analyzing the thioester bond of E1~SUMO and E2~SUMO because Coomassie Blue forms strong, but non-covalent complexes with any proteins [11,12]. Hence, to identify specific protein bands western blotting with specific antibodies against SUMO1, Uba2, and Ubc9 was used to detect protein bands [14].

The membrane was subjected to hybridizations with commercial antibodies: rabbit anti-SUMO1 (Sigma Aldrich, cat no. S8070, 1:1000), mouse anti-Uba2 (Santa Cruz, cat no. C0411, 1:1000), or rabbit anti-Ubc9 (Sigma Aldrich, cat no. U2634, 1:100) that specifically bind to the SUMO protein, Uba2 protein, and Ubc9 protein accordingly [15]. The secondary antibodies used were horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Sigma Aldrich, cat no. A9917, 1:3000) or mouse anti-rabbit IgG (Sigma Aldrich, cat no. A0545, 1:3000) and detected with Pierce ECL Western Blotting Substrate. The E1~SUMO or E2~SUMO thioester bonds can be detected by observing a 20kDa shift of the Uba2 protein bands (E1) or the Ubc9 protein bands (E2). The UVP™ machine equipped with a charge-coupled device (CCD) camera was used to capture the luminescent signals on western blots.

Analysis of western blot images

To quantify the western blot signal, we used ImageJ freeware from the NIJ to measure the relative density of each band [16]. To analyze the data, we use the band density from the sample without STE treatment as the standard. The relative density was then calculated by dividing the band density of each sample by the band density of the standard and plotted to show the dose-response relationship. STE showed a dose-dependent inhibition on the E1~SUMO thioester bond formation.

RESULTS

To determine which specific SUMOylation step is inhibited by STE, we performed E1~SUMO thioester assays in vitro. As depicted in Figure 2 [4], there were two thioester bonds that were formed before SUMO can modify its target proteins - the E1~SUMO thioester complex and the E2~SUMO thioester complex [4]. To test whether the amount of protein being used in the E1 thioester assay was sufficient, we performed the E1~SUMO thioester formation followed by SDS-PAGE and Coomassie blue staining. The Coomassie Blue stained gel has shown that the proteins of SUMO, Aos1, Uba2, and Ubc9 utilized was confirmed to be present within the protein gel (Fig. 5). In contrast to the sample with reducing reagent DTT, the samples that were stopped using non-reducing SDS loading buffer containing 8M urea showed more protein bands (Fig. 5). These extra bands were specific to the proteins of Uba2 and Ubc9, indicating that STE inhibited the thioester bond formation.
bands are the E1–SUMO and E2–SUMO thioester bands that are prone to reducing agents such as DTT. From this experiment we conclude that 1 µM of E1 and E2 enzymes in combination with 3 µM of SUMO were sufficient to perform the gel-based assay.

To obtain further evidence that the Coomassie signal observed without the DTT reducing agents reflects the E1–SUMO and E2–SUMO thioester bands, we performed western blots. As expected, there are additional bands, 20kDa shift from the Uba2 enzyme (molecular weight 80kDa) - these bands located at about 100kDa were confirmed to be the Uba2–SUMO thioester bands.

**DISCUSSION**

The FRET-based HTS to discover an inhibitor for the SUMOylation pathway was designed to discover any potential inhibitors that may inhibit any step(s) of SUMOylation pathway. Since the screening included both E1 (Aos1/Uba2) and E2 (Ubc9), it was indistinguishable as to which step in the SUMOylation pathway was inhibited by STE.

Our findings show that STE inhibits the SUMOylation pathway in regards to the E1–SUMO thioester bond formation step. Figure 6A shows that the E1–SUMO thioester band intensity decreases as the STE concentration increases from 0.1µM to 25µM. This suggests that STE inhibits the E1–SUMO thioester bond formation on a dose-dependent inhibitory relationship with the inhibitory concentration (IC₅₀) of 3.592 µM as graphed in Figure 6B.

![Fig. 6A](image)  
Fig. 6A. X Inhibits SUMO E1 thioester formation in a dose dependent manner in vitro. DMPOS or STE was pre-incubated with 1µM E1 (Aos1/Uba2) and 1µM SUMO. 2mM ATP was added to start the reaction. Reaction was incubated at 37°C for 10 min. Reactions were quenched with non-reducing sample buffer containing 4M urea, resolved by SDS-PAGE, and analyzed by immunoblotting using anti-SUMO1. A representative experiment is shown.

Fig. 6B Nonlinear regression plot of E1–SUMO thioester inhibition by STE. ImageJ software was used to compare the density of bands on western blot. The sample without STE served as the control to compare the density of STE treated samples. The values reported (mean ± SD) are an average of three independent experiments. GraphPad Prism was use to plot the data and to calculate the IC₅₀.

By inhibiting E1 (Aos1/Uba2), the first enzyme in the enzymatic reaction cascade for SUMOylation, it will ultimately result in hindering the SUMOylation pathway - preventing SUMO to undergo the enzymatic reaction cascade involving E1, E2, and E3. Hence, preventing SUMO from attaching to a specific substrate with the assistance of E3 ligases.

Since the FRET-based HTS discovered all potential inhibitors for any step in the SUMOylation pathway including both the E1(Aos1/Uba2) and E2(Ubc9), it was indistinguishable as to which step is inhibited. Our results show that STE inhibits the E1–SUMO thioester formation step. But it is possible that STE can inhibit the SUMO transfer from E1 to E2 and thus requires further determination. Given that SUMO is part of Ubiquitin and Ubiquitin-like-protein family that share similar structures and mechanisms of action, it is important to determine whether the E1–SUMO thioester formation is specifically inhibited in the SUMOylation pathway and not other ubiquitin like protein pathways – thus, determining if STE inhibition is SUMOylation specific is yet to be discovered.

Furthermore, previous reports have shown that Uba2 (E1) is required for Myc-driven cancer to survive[7]. Thus, future studies will be aimed at the potential of STE in
inhibiting Uba2 (E1) to induce Myc-driven tumor cell death.

CONCLUSION

This study provides novel insights into the mechanisms of STE as a novel small molecule inhibitor of the SUMOylation pathway. We have shown that through our FRET-based high-throughput screening method [2], we were able to identify a novel small molecule inhibitor of SUMOylation pathway. In order to understand the mechanism of this inhibition, we performed a gel-based E1–SUMO and E2–thioester assays. STE was found to inhibit the E1–SUMO thioester formation in a dose-dependent manner.

REFERENCES


