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Streptonigrin inhibits SENP1 and reduces the protein level of hypoxia-inducible factor 1 (HIF1 α) in cells

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Abstract

Streptonigrin (CAS no. 3930-19-6) is a natural product shown to have anti-tumor activities in clinical trials conducted in the 1960s–1970s. However, its use in clinical studies eventually faded, and the molecular mechanisms of streptonigrin anti-tumor effects remain poorly defined. Despite the lack of its current clinical use, efforts on its total synthesis have continued. Here, we show that streptonigrin binds and inhibits the SUMO-specific protease SENP1. NMR studies identified that streptonigrin binds to SENP1 on the surface where SUMO binds and disrupts SENP1-SUMO1 interaction. Site-directed mutations in combination with NMR chemical shift perturbation suggest key roles of aromatic pi stacking interactions in binding streptonigrin. Treatment of cells with streptonigrin resulted in increased global SUMOylation levels and reduced level of hypoxia inducible factor alpha (HIF1 α). These findings inform both the design of SENP1 targeting strategy and the modification of streptonigrin to improve its efficacy for possible future clinical use.

Graphical Abstract

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Author Contributions. Ambaye N., Chen C.-H., Li Y.-J. and S. Khanna designed and performed the experiments. Chen Y. designed the studies, analyzed data, and wrote the manuscript along with all co-authors.

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Supporting Information. Three figures include both NMR and cellular data.

INTRODUCTION

Post-translational modification with the small ubiquitin-like modifier (SUMO) proteins results in the covalent attachment of SUMO to other cellular proteins. Mammalian cells express at least three SUMO proteins, known as SUMO1, 2, and 3.^{1,2} SUMO modifications are initiated and removed by the activities of SUMO-specific proteases (SENPs) that include SENP1, SENP2, SENP3, SENP5, SENP6 and SENP7.³ SENP1 is a potential target for developing new therapeutic agents for cancer. One of its functions identified to date was regulation of the stability of hypoxia-inducible factor 1 α (HIF1 α) by removing HIF1 α SUMOylation.^{4–6} HIF1 α is a key player in angiogenesis, the formation of new blood vessels in tumors to support tumor growth.

Streptonigrin (SN, CAS no. 3930-19-6) is a natural product isolated from *Streptomyces flocculus*. In clinical trials conducted in the 1960–1970s, SN showed anti-tumor activity against various cancers, including breast, lung, head and neck, lymphoma, and melanoma.^{7–9} Despite some promising clinical results, the National Cancer Institute discontinued the supply of SN due to a lack of requests for the compound for additional clinical studies. However, laboratory studies of SN's anti-cancer effects continued and a recent study showed that SN inhibits melanoma cancer stem cells.¹⁰ In addition, several laboratories have devoted efforts to the total synthesis of SN.^{11–13} SN was recently identified as an inhibitor of protein arginine deiminases (PAD),¹⁴ which catalyze the conversion of the positively charged guanidinium group of an arginine residue to a neutral ureido-group.¹⁴ Otherwise, understanding of the molecular targets of SN is limited.

In this study, we identify SN as a small molecule inhibitor of SENPs, with higher inhibitory effect against SENP1 than other SENPs.

MATERIALS AND METHODS

Enzymatic Assays

Steady-state kinetic assays to test inhibition of SENP1 by the compounds were conducted using AMC-tagged SUMO1 as a fluorogenic substrate (Boston Biochem). The SENP1 catalytic domain (final concentration 75 pM) was incubated with the compounds at various concentrations for 10 minutes (final 0–1 μ M) in Tris buffer (50 mM Tris, pH 7.5; 150 mM NaCl; and 10 mM dithiothreitol) at room temperature (RT). Various concentrations of SUMO1-AMC (final 0–325 nM) were added to the SENP1-compound mixture for 15 minutes at RT. The reaction was measured by monitoring fluorescence at 440 nm (λ_{ex} = 340 nm) on a SpectraMax M5 plate reader. Similar experimental conditions were used for measuring the IC₅₀s of SN inhibition of the different SENPs. The kinetic data was analyzed using GraphPad Prism 6 software.

NMR experiments

The wild-type SENP1 catalytic domain was expressed and purified, and the chemical shift assignments were as described previously.¹⁵ SN was dissolved in dimethyl sulfoxide-d₆ (DMSO-d₆) and combined with SENP1 in a final buffer containing 20 mM sodium phosphate, pH 6.8; 5 mM DTT; 0.03% NaN₃; and 1% DMSO-d₆. 2D ¹H-¹⁵N correlation spectra were

acquired at 25°C on a Bruker Ascend 700 spectrometer. The data were processed with NMRPipe, and spectra were analyzed with the program SPARKY (T. D. Goddard and D. G. Kneller, SPARKY 3, University of California, San Francisco). ¹⁵N NOESY-HSQC and ¹³C NOESY-HMQC experiments were carried out with non-uniform sampling. A Possion-gap sampling schedule was generated using Schedule Generator V3.0 software (<http://gwagner.med.harvard.edu/intranet/hmsIST/index.html>).¹⁶ 3D NOESY spectra were acquired in the 300 ms mixing time with 10% of sampling density and 64×128 complex points in the indirect dimensions for ~86 h, and reconstructed by using an iterative soft thresholding approach.¹⁷ The intermolecular nuclear Overhauser effects (NOEs) between SENP1 and SN were assigned based on chemical shifts and comparing NOESY spectra for the free SENP1 and SN-bound SENP1. The ambiguous interaction restraints were used for calculation of the SN-SENP1 complex using the program HADDOCK.¹⁸ 200 best structures of the initial 1000 docking structures were selected for further semi-flexible simulated annealing and water refinement.

Site-directed mutagenesis and NMR-based binding experiments

The SENP1 mutants, F496D and W512D, were introduced onto the SENP1 C603S catalytic domain that is more stable in solution and showed similar chemical shift changes as the WT in SN-binding experiments. Mutations were confirmed by DNA-sequencing, and the mutant proteins were expressed and purified using the same approach as described in our previous publications.¹⁵ SN was dissolved in dimethyl sulfoxide-d₆ (DMS-d₆) and combined with SENP1 in the same buffer as described above for NMR studies. NMR experiments and data processing were conducted in the same way as the WT protein as described above.

Cleavage of physiological substrates of SENP1

Various concentrations of SN (0–500 μM) at RT were incubated with SENP1 for 20 min in the assay buffer (50 mM Tris, pH 7.4, 100 mM NaCl, and 10 mM DTT) before adding 1.5 μM of the final substrates. ECFP-SUMO1-YFP fusion protein and the RanGAP1-SUMO1 conjugate were made as described previously^{19, 20}. SENP1 concentrations were 15 nM for the ECFP-SUMO1-YFP fusion protein, and 5 nM for the RanGAP1-SUMO1 conjugate. The mixtures were incubated (RT, 10 mins for ECFP-SUMO1-YFP; RT, 5 mins for RanGAP1-SUMO1) and followed by SDS-PAGE and Coomassie staining for visualization. The conditions were designed to have the enzyme cleave approximately 50% substrates.

Cell culture

HCT116 colorectal cancer cells were grown in Dulbecco's Modified Eagle's medium that contained 10% heat inactivated fetal calf serum (Omega Scientific, Inc.) at 37°C, with 2 mM L-glutamine, 100 U ml⁻¹ penicillin, and 100 μg ml⁻¹ streptomycin.

Transient transfection, chemical compound treatments, and immunoprecipitation

The HIF1α expression plasmid pCAG-HIF1α was obtained from Addgene (plasmid 21101).²¹ Transient transfection of plasmid DNA was performed using transfection reagent Lipofectamine LTX (Life Technologies) per manufacturer's instructions. 24 h after transfection, cells were treated with SN (0, 1, or 2 μM) for 16 h, followed by addition of 10

μ M proteasome inhibitor MG132 for 8 h. Cells were directly lysed in 1X RIPA buffer (25 mM Tris•HCl, pH 7.6; 150 mM NaCl; 1% NP-40; 1% sodium deoxycholate; and 0.1% SDS) containing 20 mM N-Ethylmaleimide, 2 mM iodoacetamide, and 1X protease inhibitor cocktail (cOmplete EDTA-free; Roche). The cell lysate was incubated with 50 μ L of protein G-Sepharose beads for 4 hours at 4°C. After centrifugation, the supernatant was used as pre-clear fraction for immunoprecipitation. 200 μ g of each pre-cleared protein lysate was incubated with 2 μ g of rabbit anti-HIF1 α antibody (sc-10790; Santa Cruz Biotechnology) for 16 h at 4°C, followed by incubation with 20 μ L of protein G-Sepharose beads for 1 h at room temperature. After three times of washes with 1X RIPA buffer and brief centrifugations, the pull-down proteins were eluted in Laemmli buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue, and 0.125 M Tris-HCl, pH 6.8) and boiled for 5 minutes. The resulting immune complexes, as well as input protein lysates, were analyzed by SDS-PAGE separation and western blot analyses using rabbit anti-HIF1 α antibody and mouse anti-ubiquitin antibody (07–375; Millipore). GAPDH protein was visualized with goat anti-GAPDH antibody (sc-20357; Santa Cruz Biotechnology) and used for protein normalization.

To detect global SUMOylation levels, HCT116 cells, cultured at 37°C, were treated with SN at 1 or 2 μ M for 48 h; the same volume of DMSO was used as a vehicle control. The cells were harvested and directly lysed in Laemmli buffer. Lysates were separated by SDS-PAGE and SUMO1 and SUMO2/3 conjugated proteins were visualized by western blotting using antibodies against SUMO1 (C9H1; rabbit mAb #4940; Cell Signaling) or SUMO2/3 (mouse mAb #M114-3; MBL international).

For the HIF1 α stability assay, HCT116 cells were transfected with pCAG-HIF1 α for 24 h, then treated with 50 μ M of SPI-01, 2 μ M of SN, or 0.1 μ M of Carfilizomib for 16 h. The same volume of DMSO was used as a vehicle control. Treated cells were harvested, directly lysed in Laemmli buffer, separated by SDS-PAGE, and HIF1 α protein level was visualized by Western blot using rabbit anti-HIF1 α antibody.

RESULTS AND DISCUSSION

Potency and Specificity of SN for Inhibiting SENP1

To identify SENP inhibitors, we used the Glide program (Schrödinger) and the structure of SENP1/SUMO1 complex (pdb code: 2IY1)²² with SUMO1 removed, and carried out docking to screen the National Cancer Institute (NCI) diversity set IV database (1593 compounds). Then we obtained the virtual “hit” compounds from the NCI Developmental Therapeutics Program (DTP) and assayed their ability to inhibit SENPs, as described previously.¹⁵ The compounds with confirmed biochemical activity were used to search for analogs in the DTP compound repository (~260,000 structures) in an iterative fashion. The available compounds were obtained from the DTP, followed by enzymatic assays. SN was the most potent compound identified from these analyses.

We tested the effects of SN on SENP-SUMO inhibition using different combinations of SENPs and AMC-tagged fluorogenic SUMO substrates (Figure 1). SENPs form three families based on sequence and functional similarity. SENP1 and SENP2 catalyze

maturation of SUMO precursors by cleaving the C-terminal segments, resulting in a Gly-Gly sequence at the C-terminus and remove both SUMO1 and SUMO2 conjugates from target proteins. SENP3 and SENP5 remove SUMO2/3 conjugates, and SENP6 and SENP7 are mainly involved in trimming poly-SUMO2/3 chains^{23, 24} Because SUMO2 and 3 are nearly identical, SUMO2 was used as the representative isoform. When SUMO1 was used as a substrate, SN preferentially inhibited SENP1 activity ($IC_{50} = 0.518 \pm 0.100 \mu M$) compared to SENP2 activity ($IC_{50} = 6.919 \pm 0.676 \mu M$). To further determine SN's specificity, the activity of the distantly related SENP6 was tested using SUMO2-AMC as a substrate, because SENP6 does not remove SUMO1 modifications. SN inhibited SENP6 with potency similar to that of SENP2 ($IC_{50} = 5.205 \pm 0.853 \mu M$), suggesting selectivity to SENP1. These data suggest that SN has higher potency in inhibiting SENP1 relative to other SENPs. The ability of SN to inhibit SENP1 was also examined using physiological substrates, SUMO1 precursor that is fused to the ECFP at the N-terminus and YFP at the C-terminus as previously described,²² and SUMO1 conjugated RanGAP1.²⁰ Substrates and products were visualized by Coomassie staining on SDS-PAGE (Figure S1). Although the determination of the half-maximum inhibitory concentrations (IC_{50}) was hindered by accurate quantification from Coomassie-stained gels, similar potency for inhibiting SENP1 on the cleavage of peptide or isopeptide bonds was observed, because at 2 μM SN concentration, significant inhibition was observed for both substrates.

Structural Mechanism of SN Inhibition of SENP1

Next, we investigated how SN binds to SENP1 using NMR spectroscopy. 2D 1H - ^{15}N -HSQC spectra revealed that SN induces concentration-dependent NMR chemical shift perturbation (CSP) of the backbone amides of SENP1 residues K500 and T513, as well as the sidechain amide of residue W512 (Figure 2A). We used both the ^{15}N NOESY-HSQC and ^{13}C NOESY-HMQC experiments to identify intermolecular nuclear Overhauser effects (NOEs) between SENP1 and SN. The intermolecular NOEs were identified by unique chemical shifts of the compound and by comparing NOESY spectra for the free SENP1 and SN-bound SENP1 (Figure 2B). We observed intermolecular NOEs for the same area where CSP was observed, further indicating that these residues are located at the binding site (Figure 2A). SENP1 resonances were assigned, but SN resonances were not unambiguously assigned, due to resonance overlaps. Therefore ambiguous distance restraints were used for structure calculation using the program HADDOCK¹⁸ Several binding modes of SN on SENP1 were observed among the top 40 structures of the lowest total energy, suggesting that the distance constraints were not sufficient to define the binding mode of SN on SENP1. However, the top five structures (lowest total energy) showed similar binding modes (Figure 2C).

We then determined how SN interacts with the SENP1-SUMO1 complex using ^{15}N -labeled SUMO1 and unlabeled SENP1 (Figure 2D). Many SUMO1 resonances (Figure 2D, black spectrum) weakened upon complex formation with SENP1 (Figure 2D, green spectrum), due to increased NMR resonance widths as a result of increased molecular weight and due to the exchange rate of the free and bound SUMO1 in the regime similar to NMR chemical shift differences between the free and bound form. Addition of SN to the SUMO1-SENP1 complex caused the recovery of free SUMO1 resonances (Figure 2D, red spectrum),

indicating that SN disrupts the complex between SENP1 and SUMO1. The small chemical shift changes in SUMO1 are likely due to transient binding to SENP1 in the presence of SN in the equilibrium of non-covalent interactions. It is not due to SN binding to SUMO1, because titration of SN to SUMO1 directly did not result in any chemical shift changes (Figure S2).

The SN-binding site on SENP1 encompasses two segments, residues 494–500 and residues 511–512. The sidechains of Asn494, Phe496, Lys500, and Trp512 form contacts with SN. Phe496 forms parallel-displaced pi stacking interaction with the phenyl ring and hydrophobic interaction with the methyl group on the pyridyl ring (Figure 3A). Asn494 appears to form hydrogen bond with the NH₂ group on the pyridyl ring. Lys500 sidechain forms electrostatic interaction with the carboxylic acid group of SN (Figure 3A). Trp512 sidechain forms sandwich pi stacking interaction with the bicyclic quinoline ring, and Arg511 sidechain contacts the methoxy group and forms hydrogen bond with the carbonyl group (Figure 3A). The structure provides an understanding of the different inhibitory effects of SN for the structurally similar SENP1 and SENP2. Thr499 in SENP1 corresponds to a Pro in SENP2. The Pro in SENP2, which has restricted backbone conformation, may cause the differences in the orientations of residues equivalent to Asn494, Phe496 and Lys500 in SENP1, resulting in the difference in SN binding affinity.

We further validated the binding site of SN in SENP1 using site-directed mutations. F496D and W512D mutants were designed to validate aromatic pi stacking interactions in binding SN. Compared to the 2D ¹H–¹⁵N-HSQC spectra of the wild-type SENP1, both mutants did not display CSP that was observed with WT SENP1 (Figure 3B in comparison with Figure 2A). The lack of CSP implied that the mutations interfered with SN binding and that the mutated residues are critical to SN binding. These results provide validation of the calculated complex structure. We attempted to use isothermal titration calorimetry to characterize the interaction between SENP1 and SN. However, no significant heat was released or absorbed, indicating that SN-SENP1 interaction is entropy driven.

SAR Analysis of SN Inhibition

We investigated structure-activity relationship (SAR) of SN inhibition. Using enzyme kinetic analysis, we determined that SN inhibition of SENP1 exhibits a k_i of 138 ± 17.7 nM (Figure 3A and 3B). The K_m value of 61.1 ± 9.87 nM is similar to that previously reported for SENP1.²² To define SAR, we tested the inhibitory effects of similar compounds from the NCI DTP compound library. The two closest analogues of SN, NSC76919 and NSC45384, differ from SN in the aromatic ring next to the bicyclic quinoline ring and replacement of a –COOH group by an ester. These compounds also inhibited SENP1, but with a K_i approximately 2-fold higher (Figure 4A and 4B). This is consistent with the interactions observed between the SN and SENP1 (Figure 3A). Other compounds with structural similarities (Figure 4C) did not inhibit SENP1 at concentrations up to 200 μ M. Lack of inhibition by NSC62709, which lacks a quinone relative to SN, is consistent with the extensive interactions observed between the bicyclic quinoline ring and SENP1 (Figure 3A). Lack of inhibition by NSC 111567 could be due to steric hindrance caused by the bulky group attached to the bicyclic ring. These results further suggest that SN inhibition of

SENP1 is not due to non-specific effects. NMR CSP was not observed at any Cys residues (Figure 2), indicating that SN does not form a covalent adduct with SENP1. Other analogues that did not inhibit SENP are more different from SN than the above-mentioned compounds in their structures.

SN reduced HIF1 α protein level in Cells

To test the ability of SN to inhibit SENP1 in cells, we treated HCT-116 cells with 0, 1, or 2 μ M SN overnight. We lysed the cells and used Western blot to detect SUMOylated proteins. Both SUMO1 and SUMO2/3 conjugates accumulated in cells with increasing SN concentration (Figure 5A). We did not observe a significant increase in ubiquitination, indicating a lack of effect on most de-ubiquitination enzymes (Figure S3, right panel).

SUMOylation of HIF1 α induces its ubiquitination and degradation by the proteasome; SENP1 stabilizes HIF1 α by removing SUMO1 conjugation⁴ Thus, SENP1 activity is required for HIF1 α protein stability. To determine the effect of SN on a SENP1-dependent functions in cells, we examined how SN affected HIF1 α levels. We transfected HCT116 cells with a HIF1 α expression plasmid for 24 h. HIF1 α -expressing cells were treated with vehicle control, SN, or a previously identified SENP1 inhibitor (SPI-01)¹⁵ To rule out general cytotoxicity effects on HIF1 α stability, we used Carfilzomib, a proteasome inhibitor, as a control (Figure 5B). The concentration of each compound was within the range of their cellular effects on their respective targets. Both SN and SPI-01 reduced HIF1 α levels relative to DMSO-treated cells, with SN showing a more dramatic effect, consistent with the higher cellular activities of SN compared with SPI-01. Consistent with the observed effects on HIF1 α protein level, SN treatment increased modified forms as shown by higher molecular bands from immunoprecipitated HIF1 α in the absence of MG132 treatment, supporting the conclusion that HIF1 α is modified by SUMO and ubiquitin when MG132 was present to prevent degradation of the ubiquitinated HIF1 α (Figure S3). Although these data are consistent with SN inhibition of SENP1 as at least one of the mechanisms to reduce HIF1 α protein level, these data do not rule out the contribution of other mechanisms.

In summary, we have demonstrated here that SN inhibits SENP1 by disrupting SENP1-SUMO1 interaction. Supporting its role in inhibiting SENP1, SN induces accumulation of SUMOylated proteins with EC₅₀ of approximately 1 μM and induced degradation of HIF1α, a SENP1 target. HIF1α is required for the transcription of more than 60 genes involved in angiogenesis and drives the formation of new blood vessels that feed growing tumors with oxygen and nutrients and allows cancer cells to invade nearby tissues.²⁵ Because angiogenesis plays a critical role in the growth of solid tumors, it is a target for developing anti-cancer therapies. Thus, our identification of the SENP1- mediated effects of SN on both HIF1α stability and SUMOylation suggest a potential for using SN against cancer types that respond to angiogenesis inhibitors or are dependent on SUMOylation/de-SUMOylation cycles,^{26–28} including cancer stem cells.^{29, 30} The findings provided here, along with progress in the total synthesis of SN,^{11–13} also provide a basis for further modification of SN to improve its efficacy and reduce toxicity for future clinical use.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Enzyme	Substrate	IC ₅₀ (μM)
SENP1	SUMO1	0.518 ± 0.100
SENP2	SUMO1	6.919 ± 0.676
SENP6	SUMO2	5.205 ± 0.853

Figure 1. Cleavage of SUMO1-AMC or SUMO2-AMC by SENP1, SENP2, or SENP6 in the presence of increasing concentrations of SN, measured as fluorescence of free AMC. IC₅₀ shown in the bottom panel was obtained from curve fitting.

Figure 2.

NMR studies of SN interaction with SENP1. (A) A region of superimposed ^1H - ^{15}N HSQC spectra of the catalytic domain of 150 μM human SENP1 free (black) and after addition of SN at 1:1 (magenta) and 1:3 (green) molar ratio with SENP1. (B) The intermolecular NOEs between SN and SENP1 observed in ^{15}N NOESY-HSQC and ^{13}C NOESY-HMQC spectra. The chemical shifts of the backbone amides or methyl carbon are labeled. (C) The top five structures of SN-SENP1 complex are superimposed with their SENP1 structures. Due to the nearly identical SENP1 structures, only one SENP1 structure is shown in cyan and the position of catalytic Cys603 is shown in orange spheres, but all five SN structures are shown. The atoms of SN are colored according to the types: carbon (green), oxygen (red), nitrogen (blue), hydrogen (white). SUMO1 in the equivalent position in the SENP1-SUMO1 complex (pdbid: 2IY1) is shown in transparent pink. (D) A region of ^1H - ^{15}N HSQC spectrum of free ^{15}N -labeled SUMO1 (red), superimposed with the same spectral region after addition of a 1:1 ratio of SENP1 (green), and after the further addition of a 6:1 ratio of SN (black), demonstrating the reversal of SENP1-dependent broadening of SUMO1.

Figure 3. Interactions between SENP1 and SN. (A) Sidechain carbon atoms of SENP1 are shown in cyan and the residue numbers are labeled. Carbon atoms of SN is shown in green. Oxygen, nitrogen and hydrogen atoms are shown in red, blue, and white, respectively. (B) The same region of superimposed ^1H - ^{15}N HSQC spectra as shown in Figure 2A of the F496D or W512D mutants when free (black) and after addition of SN at SENP1:SN molar ratio of 1:1 (magenta) and 1:3 (green).

Figure 4. Enzyme kinetic measurements for SN and its analogs. (A) Raw data of steady-state kinetic measurements for SN and its two active analogs. (B) The data were fit to obtain the indicated kinetic parameters (K_i and K_M). (C) Structures of compounds that were chosen based on structural similarity to SN do not inhibit SENP1 and SENP2.

Figure 5. Investigation of SN function in cells. (A) Western blot showing global SUMOylation after SN treatment. HCT116 cells were treated with SN at the indicated concentrations for 48 h. Global SUMO1 and SUMO2/3 conjugations were measured by western blot using anti-SUMO1 and -SUMO2/3 antibodies. GAPDH was used as a loading control. (B) SN reduced HIF1 α level. HCT116 cells were transfected with HIF1 α expression plasmid for 24 h, then treated with indicated compounds (SPI-01, 50 μM ; SN, 2 μM ; Carfilizomib, 0.1 μM) or vehicle control (DMSO) for 16 h. Treated cells were harvested and HIF1 α protein level was detected by western blot. GAPDH was used as a loading control.