

NOTE

Kerriamycin B inhibits protein SUMOylation

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Post-translational conjugation of small ubiquitin-related modifier protein (SUMO) to protein substrates (SUMOylation) has been revealed as one of the major post-translational regulatory systems in animals and other eukaryotes. SUMO conjugation is catalyzed by a multi-step enzymatic reaction cascade similar to ubiquitinylation.¹ In the first step, the SUMO precursor is cleaved near the C-terminus by SUMO-specific proteases to expose a C-terminal diglycine. The C-terminal glycine of mature SUMO then forms a thioester linkage to the cysteine residue of SUMO-activating enzyme (E1), the Aos1/Uba2 heterodimer, to generate the E1-SUMO intermediate in an ATP-dependent manner. Next, SUMO is transferred to the active site of the cysteine residue of the SUMO-conjugating enzyme (E2), Ubc9, through another thioester bond. In the last step, E2 and the SUMO ligase (E3) catalyze SUMOylation of substrate proteins at the ϵ -amino group of internal lysine residues. Although enzymatic reactions by E1 and E2 are sufficient for catalyzing *in vitro* SUMOylation in most cases, E3s facilitate both *in vivo* and *in vitro* conjugation and are important for substrate specificity.¹

The structure of SUMO is similar to that of ubiquitin, but its functions are different. SUMOylation regulates protein subcellular localization, enzymatic activity and protein stability, which are associated with the cell cycle, transcription, DNA repair and innate immunity.^{2,3} In addition, SUMOylation has been recently linked causally to diseases, such as Alzheimer's and Huntington's diseases,⁴ viral infection⁵ and cancer.^{6,7} Notwithstanding the importance of SUMOylation in regulating diverse life phenomena and diseases, small molecule inhibitors of SUMOylation have been unexplored. Here, we report novel activity of kerriamycin B that inhibits protein SUMOylation, which will provide useful information about the role of SUMOylation in cells and drug development.

MATERIALS AND METHODS

Materials

Kerriamycin B was purified from a culture broth of an unidentified strain of actinomycetes. A goat polyclonal anti-SUMO-1 (N-19) antibody was purchased from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). A mouse monoclonal anti-T7 antibody was from Novagen (Darmstadt, Germany). Mouse monoclonal anti- α -tubulin (B-5-1-2) and anti-FLAG (M2) antibodies were purchased from Sigma (St Louis, MO, USA). Recombinant His and T7-tagged RanGAP1-C2, GST-Aos1-Uba2 fusion protein (E1), His-tagged Ubc9 (E2) and His-tagged SUMO-1 proteins were purified as previously described.⁸

Isolation of kerriamycin B

After extraction of kerriamycin B from the 4-day cultured broth filtrate with 50 ml of *n*-BuOH, the crude material was partitioned between water and EtOAc. After removal of EtOAc from the organic layer, the extract was dissolved in MeOH and subjected to ODS open column chromatography. Active substance (2 mg) was given by concentration of the fraction eluted by 80% aq. MeOH. Further purification was carried out by using preparative HPLC (Waters 600, column (Waters, Milford, MA, USA); PEGASIL ODS 20×250 mm, monitor (Senshu Scientific, Tokyo, Japan); 220 nm, mobile phase; 30% aq. CH₃CN). The active eluate at 28 min was collected to give a yellow powder (1.6 mg). HR-ESI-MS m/z^{-1} 843.34507 [M-H]⁻ (843.34392 calculated for C₄₃H₅₅O₁₇); UV λ_{\max} (MeOH) nm (ϵ) 296.0 (sh, 5595), 320.0 (4895), 422.0 (5038); [α]₅₈₉^{22+11.4} (c 0.084, MeOH); ¹H-NMR (600 MHz, CD₃OD) δ_{H} (p.p.m., J value=Hz): 7.8 (d, J =7.9, 10-H), 7.5 (d, J =7.2, 11-H), 6.89 (d, J =9.7, 6-H), 6.39 (d, J =9.3, 5-H), 5.27 (brs, J =2.0, 1''-H), 4.96 (brs, J =2>, 1''-H), 4.56 (dd, J =9.6, 1.4, 1''''-H), 4.24 (q, J =6.5, 5''-H), 3.78 (ddd, J =11.2, 8.8, 4.8, 3'-H), 3.64 (q, J =6.7, 5'''-H), 3.54 (brs, J =2>, 4''-H), 3.48 (m, 1'-H), 3.48 (m, 3''''-H), 3.48 (m, 5'-H), 3.35 (brs, 4''''-H), 3.21 (dq, J =6.2, 3.1, 5''''-H), 3.14 (d, J =9.0, 4'-H), 2.89 (q, J =9.0, 4''''-H), 2.72 (d, J =13.1, 2-Ha), 2.56 (d, J =13.1, 2-Hb), 2.54 (m, 2'-Ha), 2.18 (ddd, J =12.6, 5.0, 1.4, 2''''-Ha), 2.07 (4-Ha), 2.06 (m, 3'''-Ha), 2.04 (m, 3''-Ha), 2.04 (m, 2''-Ha), 1.98 (4-Hb), 1.94 (m, 3''-Hb), 1.88 (d, J =13.4, 2''-Ha), 1.81 (d, J =13.4, 2''''-Hb), 1.58 (m, 3'''-Hb), 1.54 (ddd, J =12.6, 5.0, 1.4, 2''''-Hb), 1.41 (m, 2''-Hb), 1.38

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(d, $J=5.8$, 6'-H), 1.28 (m, 2'-Hb), 1.24 (d, $J=6.2$, 6''''-H), 1.20 (s, 3-CH₃), 1.15 (d, $J=6.5$, 6''-H) and 0.51 (d, $J=6.5$, 6'''-H); ¹³C-NMR (150 MHz, CD₃OD) δ_C (p.p.m.): 204.9 (C₁), 189.6 (C₇), 184.1 (C₁₂), 159.0 (C₈), 146.3 (C₅), 141.5 (C_{12a}), 139.2 (C₉), 138.6 (C_{6a}), 134.3 (C₁₀), 132.4 (C_{11a}), 120.2 (C₁₁), 117.9 (C₆), 115.5 (C_{7a}), 102.8 (C_{1'''}), 95.5 (C_{1''}), 95.2 (C_{1''}), 82.7 (C_{4a}), 82.6 (C_{12b}), 78.4 (C_{4'''}), 77.78 (C_{5'}), 77.75 (C_{3'}), 77.72 (C_{4''}), 77.1 (C₃), 76.8 (C_{4'}), 73.2 (C_{5'''}), 72.35 (C_{3'''}), 72.32 (C_{1'}), 68.1 (C_{5'''}), 67.8 (C_{4'''}), 67.7 (C_{5''}), 54.7 (C₂), 44.4 (C₄), 40.6 (C_{2'''}), 37.7 (C_{2'}), 29.9 (C_{3-CH₃}), 26.3 (C_{3'''}), 25.5 (C_{2''}), 25.4 (C_{3''}), 24.1 (C_{2'''}), 18.8 (C_{6'}), 18.3 (C_{6'''}), 17.3 (C_{6''}) and 16.8 (C_{6'''}).

In vitro SUMOylation assay

The *in vitro* SUMOylation reaction was performed for 2 h at 30 °C in 20 μ l buffer (50 mM Tris (pH 7.4), 6 mM MgCl₂, 2 mM ATP and 1 mM DTT)

containing 0.1 μ g of His and T7-tagged RanGAP1-C2, 0.3 μ g of GST-Aos1/Uba2 (E1), 0.01 μ g of His-tagged Ubc9 (E2) and 0.1 μ g of His-tagged SUMO-1. Samples were separated by 10% SDS-PAGE followed by immunoblotting using an anti-T7 antibody to detect RanGAP1-C2 or an anti-SUMO-1 antibody.

Assay for SUMO-1 thioester bond formation

The reaction for the thioester bond formation was performed for 20 min at 37 °C in 20 μ l buffer (50 mM Tris (pH 7.4), 6 mM MgCl₂, 2 mM ATP) containing 1 μ g of purified GST-Aos1/Uba2 (E1) and 0.1 μ g of biotinylated SUMO-1 in the absence of DTT. The reaction was stopped by adding loading buffer without the reducing agent. Reaction products were separated by 11% SDS-PAGE and the E1-biotinylated SUMO-1 intermediate was detected by using avidin-conjugated horseradish peroxidase (Sigma).

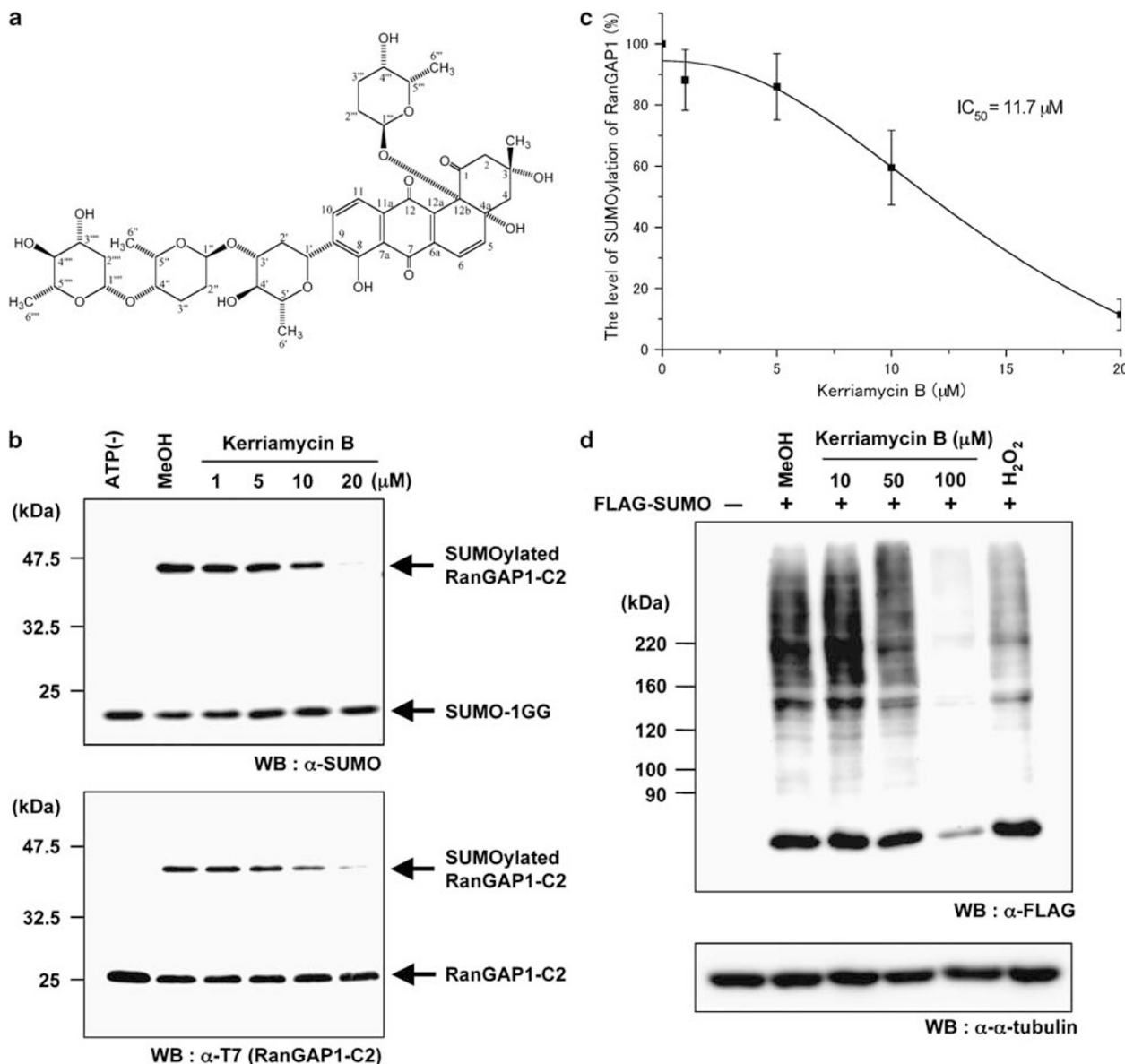


Figure 1 Kerriamycin B inhibition of protein SUMOylation. (a) Structure of kerriamycin B. (b) Dose response of kerriamycin B for SUMOylation inhibition. Indicated concentrations of kerriamycin B (1–20 μ M) were added to the SUMOylation reaction mixture containing His-tagged SUMO-1, His and T7-tagged RanGAP1-C2, the GST-Aos1-Uba2 fusion protein (E1), His-tagged-Ubc9 (E2) in the presence of 2 mM ATP. SUMOylated RanGAP1-C2 was detected by immunoblotting using an anti-T7 or an anti-SUMO-1 antibody. (c) IC₅₀ value of kerriamycin B. The level of *in vitro* SUMOylation of RanGAP1-C2 was determined by measuring the intensity of SUMOylated RanGAP1-C2 using Image Gauge Version 4.22 (FUJIFILM). The error bars show the s.d. from three independent assays and the IC₅₀ value was calculated. (d) Inhibition of *in vivo* protein SUMOylation by kerriamycin B. 293T cells were transfected with Flag-tagged SUMO and then treated with various concentrations of kerriamycin B (10–100 μ M) for 12 h or treated with 1 mM H₂O₂ for 1 h. Cells were lysed in RIPA buffer containing 50 mM *N*-ethylmaleimide, and the lysates were separated by 6% SDS-PAGE followed by immunoblotting using an anti-FLAG antibody.

Using an *in situ* cell-based SUMOylation assay method,⁹ we screened 1,839 samples of microbial cultured broth, and found extracts from three actinomycete strains showing activity to inhibit protein SUMOylation. We extracted the active substance with EtOAc from one of the extracts, and the active component was separated by using various adsorption column chromatographies. Finally, the pure compound was obtained by reverse phase HPLC. Physico-chemical properties and analysis of NMR and mass spectra showed that this compound was identical with a known antibiotic kerriamycin B (Figure 1a).¹⁰

To quantitatively analyze the SUMOylation inhibitory activity of kerriamycin B, we first characterized the effect of kerriamycin B on the *in vitro* protein SUMOylation using RanGAP1-C2 as a substrate. Kerriamycin B completely inhibited SUMOylation of RanGAP1-C2 *in vitro* at 20 μM (Figure 1b) but not *in vitro* ubiquitinylation (data not shown). The IC_{50} value of kerriamycin B against SUMOylation of RanGAP1-C2 was determined to be 11.7 μM (Figure 1c). We then asked whether kerriamycin B also inhibits *in vivo* protein SUMOylation by analyzing the effect of the level of protein SUMOylation in 293T cells expressing Flag-tagged SUMO (Figure 1d). Immunoblotting using an anti-Flag antibody showed that kerriamycin B reduced the amount of high-molecular weight SUMO conjugates at 100 μM . Treatment with hydrogen peroxide also reduced the level of high-molecular weight SUMO conjugates (Figure 1d) as recently reported.¹¹

Finally, we sought to determine the target of kerriamycin B. The complex of E1 with biotinylated SUMO-1 through the thioester bond can be detected in the presence of ATP under non-reducing conditions using a biotin-avidin detection system.⁸ The band corresponding to the E1-biotinylated SUMO-1 intermediate was detected after incubating E1 with biotinylated SUMO-1 in the presence of ATP, but this band disappeared after addition of the reducing agent DTT (Figure 2). The formation of the E1-biotinylated SUMO-1 intermediate was blocked by kerriamycin B at 20 μM (Figure 2). These results suggest that kerriamycin B inhibits protein SUMOylation by blocking the formation of the E1-SUMO-1 intermediate.

Most recently, we identified ginkgolic acid and its related compound anacardic acid present in the plant extract as the first small molecule inhibitors of protein SUMOylation.¹² Binding assays using a fluorescently labeled ginkgolic acid revealed that ginkgolic acid inhibited protein SUMOylation by directly binding to E1 to block the formation of the E1-SUMO intermediate. In this study, we rediscovered kerriamycin B as a novel inhibitor of protein SUMOylation from microbial metabolites, which also inhibited the formation of the E1-SUMO intermediate. These observations suggested that E1 is the common target for these structurally unrelated compounds.

In addition to its antibacterial activity, kerriamycin B has been shown to possess antitumor activity against Ehrlich ascites carcinoma.¹⁰ However, the mechanism underlying the antitumor activity is totally unknown. Involvement of the aberrant SUMO system in tumorigenesis has recently been suggested. The increased expression of *ubc9* encoding SUMO E2 was reported in several human ovarian cancer cell lines, such as PA-1 and OVCAR-8 as well as in ovarian tumor tissues,^{13,14} human lung adenocarcinomas,¹⁵ and LNCaP metastatic prostate cancer cell line.¹⁶ These observations might reflect a possible role of Ubc9 in tumorigenesis by regulating SUMOylation of various cellular targets. Therefore, it seems possible that kerriamycin B activity to inhibit SUMOylation is responsible, at least in part, for its antitumor activity. Further analyses on the mechanisms of SUMOylation inhibition and structure-activity relationship of kerriamycin B are necessary for developing a novel anticancer agent targeting aberrant protein SUMOylation.

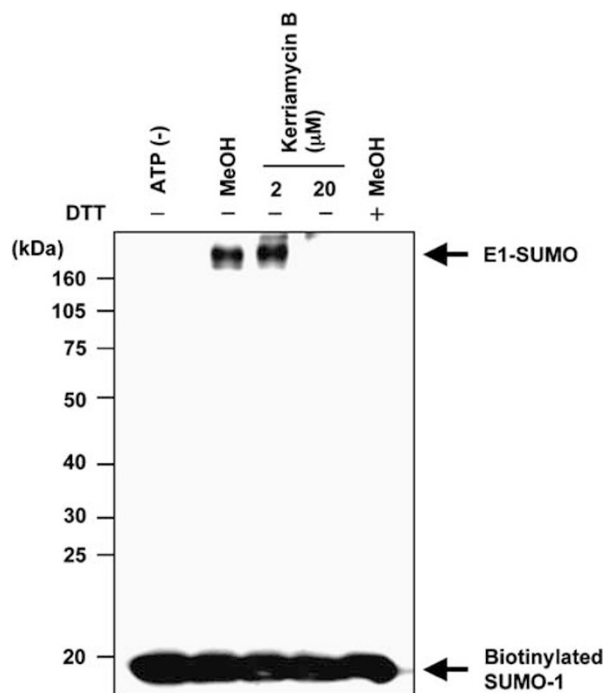


Figure 2 Impairment of the thioester bond formation between E1 and biotinylated SUMO-1 by kerriamycin B. Indicated concentration of kerriamycin B was added to a reaction mixture containing 5 $\text{ng}\mu\text{l}^{-1}$ of biotinylated SUMO-1 and 50 $\text{ng}\mu\text{l}^{-1}$ of GST-Aos1/Uba2 in the presence or absence of 2 mM ATP. After the mixtures had been incubated at 37 °C for 20 min, they were separated by SDS-PAGE, followed by analysis using avidin-conjugated horseradish peroxidase. Addition of 1 mM DTT to the reaction completely abolished the complex formation of biotinylated SUMO-1 and GST-Aos1/Uba2.

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