Kerriamycin B inhibits protein SUMOylation

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Abstract

Protein SUMOylation has recently been shown as one of the major post-translational modifications involved in a variety of signaling pathways in cells. In the course of our screening program using an *in situ* SUMOylation-detecting system, kerriamycin B, which had been originally isolated as an antibiotic, was identified as a novel inhibitor of protein SUMOylation. Kerriamycin B inhibits both *in vitro* and *in vivo* protein SUMOylation by blocking the formation of the E1-SUMO intermediate. Our findings will provide not only a novel tool for investigating the role of SUMO conjugations but also useful information for developing therapeutic drugs.

Keywords

SUMO-1, SUMO-activating enzyme (E1), Kerriamycin B

Introduction

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. While 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 infection [5], 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. A mouse monoclonal anti-T7 antibody was from Novagen. Mouse monoclonal anti-α-tubulin (B-5-1-2) and anti-FLAG (M2) antibodies were purchased from Sigma. 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; PEGASIL ODS 20 x 250 mm, monitor; 220 nm, mobile phase; 30% aq. CH_3CN). The active eluate at 28 min was collected to give a yellow powder (1.6 mg). HR-ESI-MS m/z 843.34507 $[M-H]^-$ (843.34392 calcd for $C_{43}H_{55}O_{17}$); UV λ_{max} (MeOH) ^{nm} (ε) 296.0 (sh, 5595), 320.0 (4895), 422.0 (5038); $[\alpha]_{589}^{22}$ + 11.4° (c 0.084, MeOH); ^{1}H-NMR (600 MHz,

CD_3OD) δ_H (ppm, 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 (d, *J* = 5.8, 6’-H), 1.28 (m, 2’-Hb), 1.24 (d, *J* = 6.2, 6””-H), 1.20 (s, 3-CH_3), 1.15 (d, *J* = 6.5, 6”-H), and 0.51 (d, *J* = 6.5, 6”’-H); ^{13}C-NMR (150MHz, CD_3OD) δ_C (ppm) : 204.9 (C_1), 189.6 (C_7), 184.1 (C_{12}), 159.0 (C_8), 146.3 (C_5), 141.5 (C_{12a}), 139.2 (C_9), 138.6 (C_{6a}), 134.3 (C_{10}), 132.4 (C_{11a}), 120.2 (C_{11}), 117.9 (C_6), 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 (C_3), 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 (C_2), 44.4 (C_4), 40.6 ($C_{2''''}$), 37.7 ($C_{2'}$), 29.9 ($C_{3\text{-}CH3}$), 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 μl buffer (50 mM Tris (pH 7.4), 6 mM $MgCl_2$, 2 mM ATP, and 1 mM DTT) containing 0.1 μg of His and T7-tagged RanGAP1-C2, 0.3 μg of GST-Aos1/Uba2 (E1), 0.01 μg of His-tagged Ubc9 (E2), and 0.1 μg 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 μl buffer (50 mM Tris (pH 7.4), 6 mM $MgCl_2$, 2 mM ATP) containing 1 μg of purified GST-Aos1/Uba2 (E1), and 0.1 μg 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).

Results and Discussion

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 (Fig. 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. Kerrriamycin B completely inhibited SUMOylation of RanGAP1-C2 *in vitro* at 20 μM (Fig. 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 (Fig. 1C). We then asked whether kerriamycin B also inhibits *in vivo* protein SUMOylayion by analyzing the effect of the level of protein SUMOylation in 293T cells expressing Flag-tagged SUMO (Fig. 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 (Fig. 1D) as recently reported [11].

Finally, we sought to determine the target of kerriamycin B. The complex of E1 with biotinylated SUMO-1 via the thioester bond can be detected in the presence of ATP under nonreducing 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 (Fig. 2). The formation of the E1-biotinylated SUMO-1 intermediate was blocked by kerriamycin B at 20 μM (Fig. 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 metastastic 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.

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Figure legends

Fig. 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 μM) 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. SUMOlylated RanGAP1-C2 was detected by immunoblotting using an anti-T7 or an anti-SUMO-1 antibody. (**C**) IC_{50} 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 standard deviations from three independent assays and the IC_{50} 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 μM) for 12 h or treated with 1 mM H_2O_2 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.

Fig. 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 ng/μl of biotinylated SUMO-1 and 50 ng/μl 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.

Fig. 1

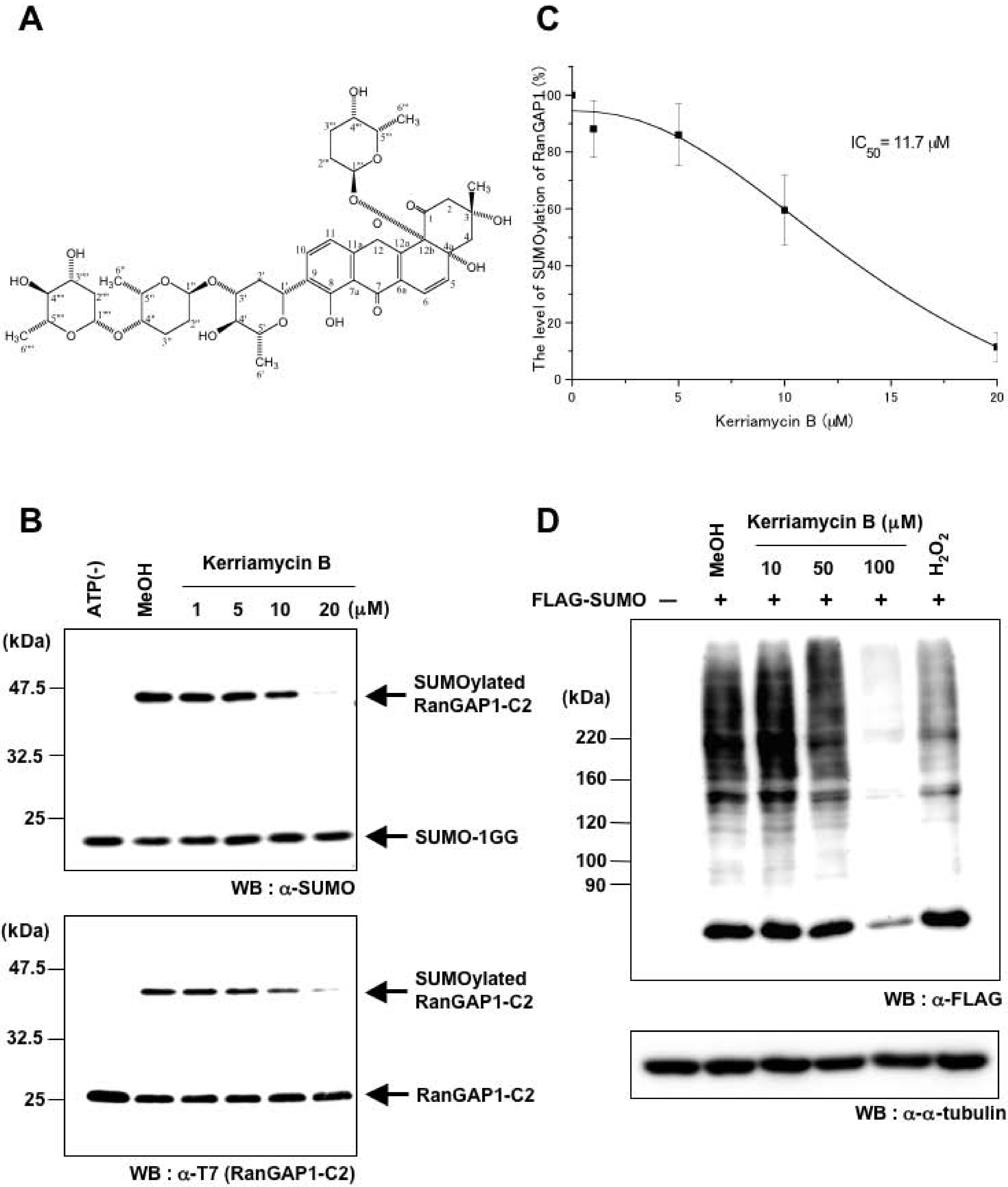
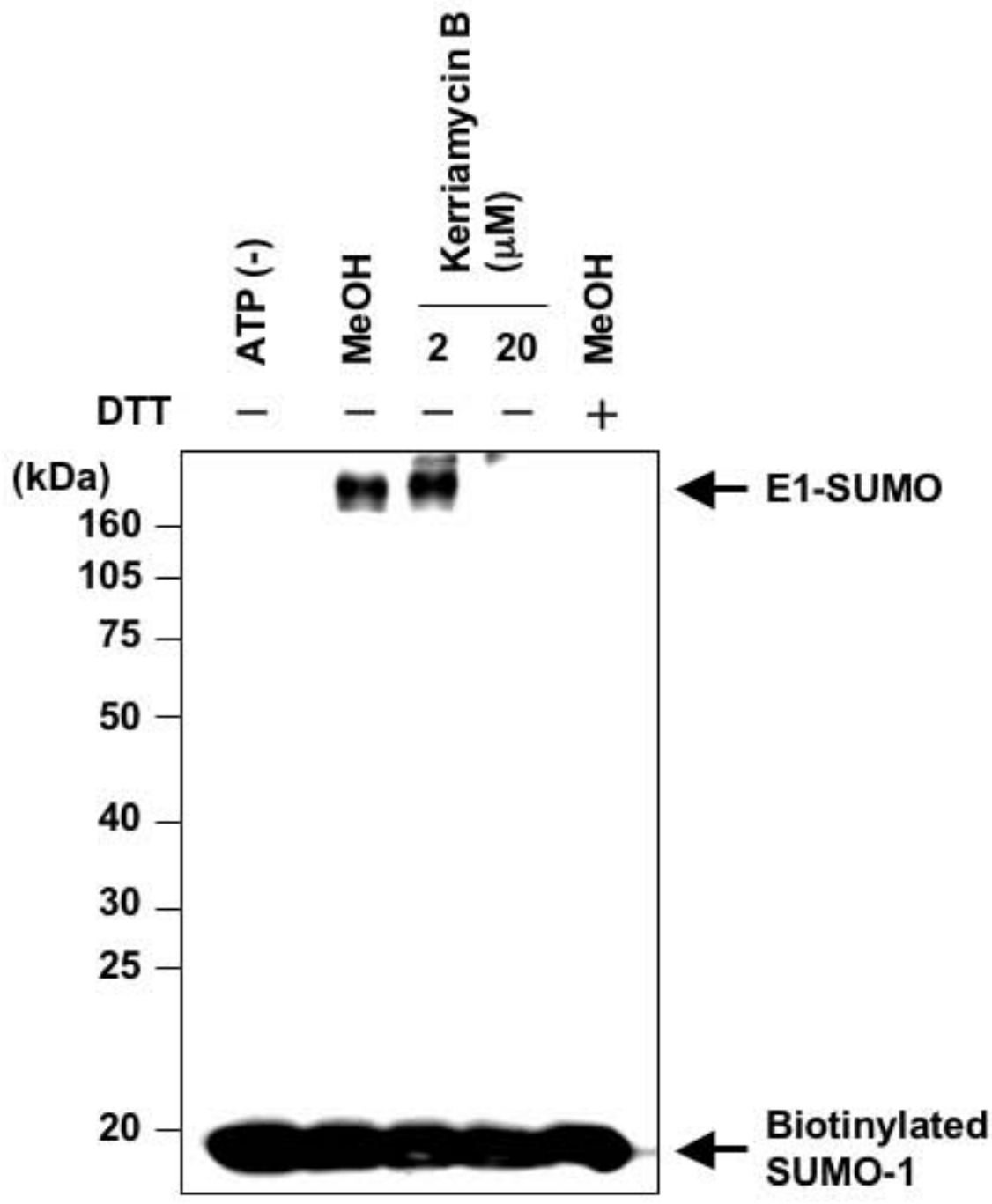

A
C
The level of SUMOylation of RanGAP1 (%)
IC_{50} = 11.7 μM
Kerriamycin B (μM)
B
ATP(-)
MeOH
Kerriamycin B
1
5
10
20 (μM)
(kDa)
47.5
32.5
25
SUMOylated RanGAP1-C2
SUMO-1GG
WB : α-SUMO
(kDa)
47.5
32.5
25
SUMOylated RanGAP1-C2
RanGAP1-C2
WB : α-T7 (RanGAP1-C2)
D
Kerriamycin B (μM)
MeOH
10
50
100
H_2O_2
FLAG-SUMO — + + + + +
(kDa)
220
160
120
100
90
WB : α-FLAG
WB : α-α-tubulin

Fig. 2


ATP (-)
MeOH
Kerriamycin B (μM)
2
20
MeOH
DTT
–
–
–
–
+
(kDa)
160
105
75
50
40
30
25
20
E1-SUMO
Biotinylated SUMO-1