NOTE

Inhibition of protein SUMOylation by natural quinones

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Conjugation of small ubiquitin-related modifier (SUMO) to target proteins (SUMOylation) is a posttranslational modification that regulates a wide range of biological processes, including transcription, intracellular transport, DNA repair, DNA replication and cell signaling.¹ SUMOylation is catalyzed by a multi-step enzymatic cascade that resembles ubiquitination: the process involves an E1-activating enzyme (a heterodimer consisting of Aos1 and Uba2), an E2-conjugating enzyme (Ubc9) and various E3 ligases.¹ E3s catalyze SUMO conjugation to target proteins and may contribute to substrate specificity. SUMO is subsequently cleaved from target proteins by SUMO-specific isopeptidases. Because SUMO modifications have been implicated in a variety of disorders such as cancers² and neurodegenerative diseases,³ the enzymes involved in SUMOylation are promising targets for therapeutic agents.

Several small-molecule inhibitors of SUMOylation have been identified. The first class of SUMOylation inhibitors to be characterized were ginkgolic acid and anacardic acid, which are isolated from the extract of *Ginkgo biloba* leaves.⁴ Subsequently, natural products, including two antibiotics (kerriamycin B⁵ and spectomycin B¹⁶) and davidiin from the plant *Davidia involucrata*,⁷ as well as synthetic compounds,^{8–11} have been shown to inhibit protein SUMOylation. In this study, we identified another class of natural products, which includes nocardione A and β -lapachone, that act as SUMOylation inhibitors. The results of mode-of-action studies suggested that these quinone-related compounds inhibit SUMOylation by oxidizing thiols of E1 and E2 enzymes through reactive oxygen species (ROS).

The materials for this study were obtained as follows. Goat polyclonal anti-SUMO-1 (N-19) and anti-UBC9 (N-15) antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Mouse monoclonal anti-T7 antibody was purchased from Merck Millipore (Billerica, MA, USA). Mouse monoclonal anti- α -tubulin (B-5-1-2) and anti-FLAG (M2) antibodies were purchased from Sigma (St Louis, MO, USA). CM-H2DCFDA was purchased from Life Technologies (Carlsbad, CA, USA). Plasmids for expression of His-tagged SUMO-1, His- and T7-tagged RanGAP1-C2, GST-Aos1/

Uba2, and His-tagged Ubc9 plasmids and biotinylated SUMO-1 were described previously.⁴ 293T, HeLa, and A549 cells were maintained at 37 °C in 5% CO₂ in Dulbecco's modified Eagle's medium supplemented with L-glutamine and 10% fetal bovine serum (GIBCO, Carlsbad, CA, USA). *In vitro* and *in vivo* SUMOylation assays were performed as described.⁴ The measurement of intracellular ROS was performed as follows. A549 cells suspended in phosphate-buffered saline were incubated with 10 μ M CM-H2DCFDA for 15 min at 37 °C, and then equal number of cells were distributed to the 96-well plate. After incubation for 30 min at 37 °C, the fluorescence intensity was measured at λ_{ex} 488 nm/ λ_{em} 520 nm with a SpectraMax M2 microplate reader (Molecular Devices, Sunnyvale, CA, USA). Assay for dithiothreitol (DTT)-sensitive crosslinking between E1 and E2 was performed as previously described.¹²

In situ cell-based screening⁴ of small-molecule libraries identified several compounds that inhibit SUMOylation, including four chemicals with ortho-quinone in their structures. These hit compounds included nocardione A, originally isolated as a tyrosine phosphatase inhibitor with antifungal activity,¹³ and its analogs 3,3-DINOR-dunnione, 3,3-DINOR-dehydrodunnione and β -lapachone, which were originally isolated from the bark of the lapacho tree (Figure 1a).¹⁴ *In vitro* SUMOylation assay confirmed that these four ortho-quinone-containing compounds inhibited *in vitro* protein SUMOylation in a dose-dependent manner (Figure 1b). The 50% inhibitory concentration (IC₅₀) values of nocardione A, 3,3-DINOR-dunnione, 3,3-DINOR-dehydrodunnione and β -lapachone were 3.9, 1.1, 1.2 and 0.4 μ M, respectively (Figure 1c).

We next asked whether these quinone-containing compounds could inhibit *in vivo* protein SUMOylation by analyzing their effects in 293T cells expressing FLAG-tagged SUMO-1 (Figure 1d). Immunoblotting with an anti-FLAG antibody revealed that all compounds reduced the levels of high-MW SUMO conjugates at concentrations in the range 10–50 μ M. The relatively weak *in vivo* inhibition of protein SUMOylation by nocardione A may reflect its relatively high *in vitro* IC₅₀ and/or its relatively poor membrane permeability ascribable to a

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Figure 1 Quinone-related compounds inhibit protein SUMOylation. (a) Structure of nocardione A, 3,3-DINOR-dunnione, 3,3-DINOR-dehydrodunnione and β-lapachone. (b) Inhibition of *in vitro* SUMOylation by quinone-related compounds. Compounds at the indicated concentrations (0.1–20 μM) were added to a reaction mixture containing His-tagged SUMO-1, His- and T7-tagged RanGAP1-C2, GST-Aos1/Uba2 (E1) and His-tagged-Ubc9 (E2) in the presence of ATP. SUMOylated RanGAP1-C2 was detected by immunoblotting using an anti-T7 or anti-SUMO-1 antibody. (c) IC₅₀ values of compounds for inhibition of SUMOylation of RanGAP1-C2 *in vitro*. The level of SUMOylation of RanGAP1-C2 was determined by measuring the intensity of the band corresponding to SUMOylated RanGAP1-C2, using Image Gauge version 4.22 (FUJIFILM, Tokyo, Japan). Error bars show the s.d.'s from three independent assays, and the IC₅₀ value was calculated by comparison with a control sample incubated without the test compounds (the control SUMOylation level was defined as 100%). (d) Inhibition of *in vivo* SUMOylation by quinone-related compounds. 293T cells were transfected with FLAG-tagged SUMO, and then treated with various concentrations of compounds (1–50 μM) for 6 h or 1 mM H₂O₂ for 1 h. Cells were lysed in radioimmunoprecipitation assay buffer containing 50 mM *N*-ethylmaleimide, and the lysates were separated by 6% SDS-polyacrylamide gel electrophoresis followed by immunoblotting with anti-FLAG antibody.

phenolic hydroxyl group in nocardione A. These results indicated that nocardione A and its derivatives 3,3-DINOR-dunnione, 3,3-DINOR-dehydrodunnione and β-lapachone are a novel class of SUMOylation inhibitors.

Because these natural products share ortho-quinone as a common skeleton in their structures (Figure 1a), ortho-quinone may be responsible for inhibition of SUMOylation. Quinones are highly

redox-active molecules that generate ROS, including hydrogen peroxide, superoxide and hydroxyl radical.¹⁵ Indeed, nocardione A generated intracellular ROS in a dose-dependent manner (Figure 2a). Notably in this regard, ROS such as hydrogen peroxide have been shown to inhibit SUMO conjugation.¹² Consistent with this previous observation, treatment with hydrogen peroxide reduced the level of high-MW SUMO conjugates in cells (Figure 1d). Accordingly,

Figure 2 Quinone-related compounds inhibit SUMOylation through reactive oxygen species (ROS) formation. (a) Generation of ROS by nocardione A. A549 cells were treated with nocardione A at the indicated concentrations for 30 min and the level of intracellular ROS was measured using CM-H₂DCFDA. (b) Quinone-related compounds induce dithiothreitol (DTT)-sensitive crosslinking between E1 and E2 proteins. Either 1 mM H₂O₂ or 10 μM of the indicated compound was added to a reaction mixture containing 50 ng μl⁻¹ of GST-Aos1/Uba2 or GST-Aos1/Uba2 C173S mutant, and 25 ng μl⁻¹ of His-tagged-Ubc9 in the presence (upper panel) or absence (lower panel) of DTT. After incubation at 37 °C for 20 min, the proteins were separated by SDS-polyacrylamide gel electrophoresis, followed by immunoblotting with anti-Ubc9 antibody. (c) Inhibition of SUMOylation by compounds was counteracted by antioxidant *N*-acetyl-L-cysteine (NAC) *in vitro*. Compounds at the indicated concentrations were incubated with the SUMOylation reaction mixture with or without 5 mM NAC, and then SUMOylated RanGAP1-C2 was detected as described in Figure 1b.

we asked whether inhibition of protein SUMOylation by these quinone-related compounds is mediated by ROS generated from the quinone moieties in their structures. To explore this possibility, we first tested the effects of these compounds on disulfide formation between E1 and E2 because hydrogen peroxide generates a DTT-sensitive crosslink between E1 and E2 in the absence of ATP.¹² For this purpose, wild-type or C173S mutant E1 and E2 proteins were incubated with or without the quinone compounds, or hydrogen peroxide in the absence of ATP, followed by immunoblotting with anti-Ubc9 antibody. In agreement with the previous report,¹² DTT-sensitive high-MW species, probably attributable to the formation of a disulfide bond between E1 and E2, appeared following treatment with hydrogen peroxide (Figure 2b). It is noteworthy that these DTT-sensitive high-MW species were also detected in the presence of all quinone-containing compounds (Figure 2b), suggesting that these compounds induce a disulfide crosslink between E1 and E2. As the C173S mutant could also form the high-MW species, it seems possible that disulfide formation occurs independently of the catalytic cysteine residue. Finally, we examined effects of the antioxidant *N*-acetyl-L-cysteine (NAC) on the inhibition of *in vitro* SUMOylation by quinone-containing compounds. As shown in Figure 2c, all quinone-containing compounds inhibited SUMOylation of RanGAP1-C2 as expected, but their inhibitory effects were counteracted by NAC.

This study uncovered novel physiological activities of natural quinones, including nocardione A and its derivatives 3,3-DINOR-dunnione, 3,3-DINOR-dehydrodunnione and β -lapachone, revealing them as a novel class of SUMOylation inhibitors. Our results suggest the involvement of ROS, possibly generated by the quinone moiety, in the inhibition of SUMOylation by these compounds. Although the precise mechanism by which quinone-related compounds inhibit protein SUMOylation remains to be elucidated, our findings suggest that this inhibitory activity may be a general pharmacological activity of small-molecule compounds containing quinone in their structures. Consistent with this, kerriamycin B, an antibiotic reidentified as a SUMOylation inhibitor, also contains quinone in its structure.⁵

Both nocardione A and β -lapachone exhibit antiproliferative activities in cancer cells.^{13,14} Because recent studies implicated aberrant SUMO machinery in cancer development, the antitumor activity of quinone-containing compounds may be attributable, at least in part, to their ability to inhibit SUMO conjugation, in addition to the DNA damage induced through ROS formation.¹⁶ ARQ761, a clinical form of β -lapachone, is currently undergoing a clinical trial in patients with

advanced solid tumors. Thus, these simple natural quinones are good starting materials for development of anti-cancer drugs.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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