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NOTE

Inhibition of protein SUMOylation by davidiin, an ellagitannin from *Davidia involucrata*

Misao Takemoto[1,2], Yumi Kawamura[3], Mikako Hirohama[4,5,6], Yuki Yamaguchi[2], Hiroshi Handa[7], Hisato Saitoh[8], Yoichi Nakao[5], Manabu Kawada[9], Khan Khalid[4], Hiroyuki Koshino[10], Ken-ichi Kimura[3], Akihiro Ito[4,11] and Minoru Yoshida[1,4,6,11]

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Conjugation of small ubiquitin-related modifier (SUMO) to lysine residues in target proteins is a multistep enzymatic reaction analogous to ubiquitination.[1] Protein SUMOylation regulates numerous biological processes including transcription, the cell cycle, DNA repair and innate immunity.[1] In the first step of the reaction, SUMO is cleaved from the SUMO precursor by SUMO-specific proteases. Next, SUMO is bound to the cysteine residue of the SUMO-activating enzyme (E1), forming a thioester linkage in an ATP-dependent manner. SUMO is then transferred from E1 to the cysteine residue of the SUMO-conjugating enzyme (E2). Finally, SUMO ligase (E3) catalyzes the SUMOylation of specific substrates via a direct interaction with E2 and the substrates. Like ubiquitination, SUMOylation is reversible; the deSUMOylation process is mediated by SUMO-specific proteases. Abnormal SUMOylation is implicated in various diseases including neurodegenerative disease,[2] viral infection[3] and cancer.[4,5] Therefore, enzymes responsible for the SUMO conjugation pathway represent potential targets for drug discovery.

To date, several natural products including ginkgolic acid,[6] anacardic acid,[6] kerriamycin B[7] and spectomycin B1[8] as well as synthetic compounds,[9] have been reported to inhibit protein SUMOylation. Here, we report another natural product that functions as a SUMOylation inhibitor: davidiin, purified from the plant *Davidia involucrata*. Although most known SUMOylation inhibitors function in the micromolar range, davidiin is particularly potent, inhibiting at sub-micromolar concentrations.

Materials for this study were obtained as follows. Goat polyclonal anti-SUMO-1 (N-19) and goat polyclonal anti-p53 (FL393)-G antibodies were 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 described previously.[10] 293T, H1299, MKN-45, DU-145 and NCI-H460 cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% FBS at 37 °C under 5% CO_2.

The *in vitro* SUMOylation reaction was performed as described.[6] Briefly, *in vitro* SUMOylation reaction was performed for 2 h at 30 °C in 20 μl buffer (50 mM Tris-HCl (pH 7.4), 6 mM $MgCl_2$, 2 mM ATP and 1 mM dithiothreitol) containing His- and T7-tagged RanGAP1-C2, GST-Aos1/Uba2 (E1), His-tagged Ubc9 and His-tagged SUMO-1. Samples were separated by 10% SDS–PAGE followed by immunoblotting using an anti-T7 antibody and an anti-SUMO-1 antibody.

The reaction for thioester bond formation between SUMO and E1 was performed as described.[6] Briefly, the reaction for the thioester bond formation was performed for 20 min at 37 °C in 20 μl buffer (50 mM Tris-HCl (pH 7.4), 6 mM $MgCl_2$, 2 mM ATP) containing GST-Aos1/Uba2 (E1) and biotinylated SUMO-1 in the absence of dithiothreitol. Samples were separated by 11% SDS–PAGE and the E1–biotinylated SUMO-1 intermediate was detected by avidin-conjugated horseradish peroxidase (Sigma).

A screen of 750 samples of botanical and food ingredients extracts using an *in situ* cell-based SUMOylation assay[11] revealed several samples that could inhibit protein SUMOylation, including an extract of *D. involucrata* (data not shown).[6] The inhibitory activity of the *D. involucrata* extract was confirmed by *in vitro* SUMOylation assay using RanGAP1-C2 as substrate (Figure 1a). Compound A was isolated by activity-guided fractionation and it was identified by

[1]Drug Discovery Platform Cooperation Division, RIKEN Center for Sustainable Resource Science, Wako, Japan; [2]Department of Biological Information, Tokyo Institute of Technology, Yokohama, Japan; [3]Graduate School of Agriculture, Iwate University, Morioka, Japan; [4]Chemical Genetics Laboratory, RIKEN, Wako, Japan; [5]Department of Chemistry and Biochemistry, Waseda University, Tokyo, Japan; [6]Japan Science and Technology Corporation, CREST Research Project, Kawaguchi, Japan; [7]Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, Yokohama, Japan; [8]Department of New Frontier Sciences, Graduate School of Science and Technology, Kumamoto University, Kumamoto, Japan; [9]Institute of Microbial Chemistry (BIKAKEN), Numazu, Microbial Chemistry Research Foundation, Numazu, Japan; [10]Global Research Cluster, RIKEN, Wako, Japan and [11]Chemical Genomics Research Group, RIKEN Center for Sustainable Resource Science, Wako, Japan
Correspondence: Dr A Ito, Chemical Genetics Laboratory, RIKEN, 2-1 Hirosawa, Wako 351-0198, Japan.
E-mail: akihiro-i@riken.jp
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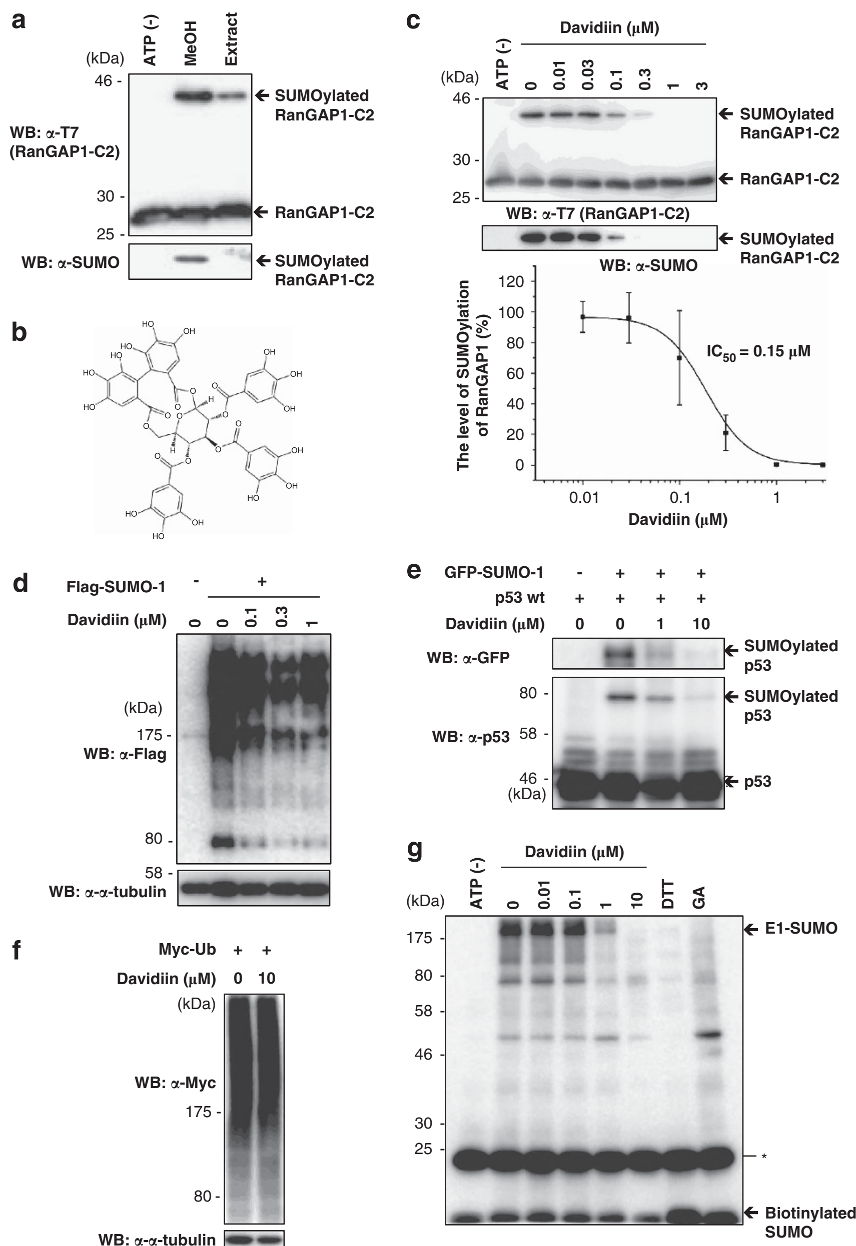

Figure 1 Davidiin inhibits protein SUMOylation. (**a**) Inhibition of protein SUMOylation by MeOH extracts of *Davidia involucrata*. *D. involucrata* extract ($1\,\mu g\,ml^{-1}$) was added to a SUMOylation reaction mixture and SUMOylated RanGAP1-C2 was detected by immunoblotting using an anti-T7 or anti-SUMO-1 antibody. (**b**) Structure of davidiin. (**c**) Dose response of davidiin for SUMOylation inhibition. SUMOylated RanGAP1-C2 was detected as described in **a**. The intensity of SUMOylated RanGAP1 was measured using an Image Gauge Version 4.22 (Fujifilm). Error bars show the standard deviations from three independent assays, and the IC_{50} value was calculated. (**d**) Inhibition of *in vivo* SUMOylation by davidiin. 293T cells expressing FLAG-tagged SUMO-1 were treated with the indicated concentrations of davidiin for 12 h and cell lysates were separated by 6% SDS–PAGE, followed by immunoblotting using anti-FLAG or anti–α-tubulin antibody. (**e**) Effects of davidiin on p53 SUMOylation. H1299 cells ($p53^{-/-}$) were transfected with p53 alone or with GFP-fused SUMO-1 and then treated with davidiin for 24 h. The immune complex obtained with an anti-p53 (FL393)-G antibody was analyzed by immunoblotting using an anti-GFP or an anti-p53 (Ab-6) antibody. (**f**) Effects of davidiin on *in vivo* ubiquitination. 293T cells expressing Myc-tagged ubiquitin were treated with 10 μM of davidiin for 12 h in the presence of 10 μM of the proteasome inhibitor MG132. Cell lysates were separated by 6% SDS–PAGE, followed by immunoblotting using an anti-Myc antibody. (**g**) Inhibition of the thioester bond formation between E1 and biotinylated SUMO-1 by davidiin. Addition of 1 mM dithiothreitol or ginkgolic acid (GA, 10 μM) as a positive control[6] abolished the formation of the E1–biotinylated SUMO-1 intermediate. The asterisk represents a non-specific band.

mass spectrometry (MS) (JMS-700, JEOL Ltd, Tokyo, Japan), NMR (EX-400, JEOL Ltd), UV (UV mini 1240, Shimadzu Co., Kyoto, Japan), and optical rotation (DIP1000, JASCO Co., Tokyo, Japan). Briefly, leaves of *D. involucrata* (328.3 g) were harvested from the Botanical Gardens of Iwate University and dried naturally at room temperature. The dried leaves (113.0 g) were extracted with MeOH, and the extract was diluted with water. The MeOH extract (34.05 g) was extracted with ethyl acetate (1 vol, two times) and evaporated (8.96 g). Half of the ethyl acetate extract was subjected to a Diaion HP-20 column (3 cm diameter × 20 cm) and an active fraction was

eluted with 50% MeOH two times repeatedly (1.06 g). Biologically active peak A was isolated from part of the 50% MeOH fraction (100.0 mg) using an HPLC system (880-PU (pump) equipped with an MD-910 photodiode array detector (JASCO, Tokyo, Japan)) on a Capcell Pak ODS (20 mm diameter × 250 mm; Shiseido Inc., Tokyo, Japan) with 15% CH_3CN–0.1% CH_3COOH at a flow rate of 5 ml min^{-1}. After the peak A fraction was isolated, it was subjected to HP-20 column (2.1 cm diameter × 7.0 cm) and eluted with 50% MeOH. The product was obtained as a light brownish powder after lyophilization (18.0 mg).

Data obtained were as follows: HR-FAB-MS *m/z* $(M+H)^+$, calculated for $C_{41}H_{31}O_{26}$, 939.1104; found, 939.1152; ^{1}H NMR δ_H of the glucose moiety of compound A (400 MHz, acetone-d$_6$) 6.13 (1H, d, $J=2.7$), 5.53 (1H, dd, $J=2.7$, 7.0), 5.79 (1H, dd, $J=6.5$, 7.0), 5.21 (1H, dd, $J=2.7$, 6.5), 4.57 (1H, ddd, $J=2.7$, 5.1, 12.1), 4.84 (1H, dd, $J=11.7$, 12.1), 4.42 (1H, dd, $J=5.1$, 11.7), ^{13}C NMR δ_C (100 MHz, acetone-d$_6$) 94,1 (C-1), 70.2 (C-2), 68.7 (C-3), 70.7 (C-4), 74.9 (C-5), 64.8 (C-6); the UV spectrum (nm) (ε) in MeOH 279 (49800); $[\alpha]_D^{25}=+20.2°$ ($c=0.2$, MeOH). All physicochemical properties were identical with data reported for a known ellagitannin, davidiin, previously isolated from *D. involucrata* (Figure 1b).[12–14] Purified davidiin inhibited *in vitro* SUMOylation of RanGAP1-C2 in a dose-dependent manner, and its IC_{50} value was 0.15 μM (Figure 1c). We next examined whether davidiin affects *in vivo* protein SUMOylation by analyzing the levels of SUMOylated proteins in 293T cells expressing FLAG-tagged SUMO-1. Immunoblotting using an anti-FLAG antibody revealed that davidiin reduced the amount of high-molecular weight SUMO conjugates in a dose-dependent manner (Figure 1d). In addition, davidiin reduced the level of SUMOylation of p53 in a dose-dependent manner (Figure 1e). Importantly, davidiin did not influence the cellular level of ubiquitinated proteins (Figure 1f). These results indicate that davidiin inhibits protein SUMOylation both *in vitro* and *in vivo* without affecting protein ubiquitination. Next, we sought to identify the target of davidiin. The formation of an E1 conjugate to biotinylated SUMO-1 via a thioester linkage could be detected in the presence of ATP under non-reducing conditions; the band corresponding to the E1–biotinylated SUMO-1 intermediate disappeared when the reducing agent dithiothreitol or the SUMO E1 inhibitor ginkgolic acid[6] was added to the reaction mixture (Figure 1g). The formation of an intermediate between E1 and biotinylated SUMO-1 was also blocked by davidiin in a dose range similar to that required for inhibition of *in vitro* SUMOylation (Figures 1c and g). These results suggest that davidiin inhibits protein SUMOylation by inhibiting the formation of the E1–SUMO-1 intermediate.

High-level expression of genes involved in SUMOylation was frequently observed in cancer. In addition, SUMO E1 has an important role in Myc-driven tumorigenesis.[15] Accordingly, we tested effects of davidiin on proliferation of cancer cells. As shown in Figure 2, davidiin inhibited cell proliferation of several cancer cells in a dose-dependent manner with similar GI_{50} values (gastric cancer MKN-45 cells: 8.3 μM, prostate cancer DU-145 cells: 11.6 μM, lung cancer NCI-H460 cells: 16.4 μM).

Davidiin is an ellagitannin previously shown to inhibit the binding of a ligand to a μ-opioid receptor.[16] In this study, we demonstrated a novel physiological activity of davidiin, namely, inhibition of protein SUMOylation. The IC_{50} value of davidiin against *in vitro* protein SUMOylation is 0.15 μM (Figure 1c), the most potent among small-molecule SUMOylation inhibitors reported to date.[6–9] Recent studies have shown that davidiin inhibits growth of hepatocellular carcinoma cells by downregulating EZH2,[17] the enzymatic subunit of the Polycomb-repressive complex 2 that catalyzes histone H3 lysine 27 methylation, which is highly expressed in a variety of human cancers. Importantly in this regard, EZH2 is SUMOylated in cells.[18] In this study, we showed that davidiin inhibited cell growth of several cancer cells including DU-145 cells, in which EZH2 is overexpressed (Figure 2).[19] Importantly, concentrations of GI_{50} values of davidiin for these cancer cells were sufficient to remarkably inhibit protein SUMOylation in cells (Figures 1d and e). Although the molecular mechanism by which davidiin inhibits cancer cell growth remains to be elucidated, these observations suggested that the anti-tumor activity of davidiin is mediated, at least in part, by inhibition of SUMOylation of proteins including EZH2. The total synthesis of davidiin was recently established by Kasai *et al.*[20] Because davidiin is the strongest natural SUMOylation inhibitor reported so far, it is an excellent starting material for drug discovery. Analyses of the structure–activity relationship of this compound should facilitate development of novel anticancer agents targeting protein SUMOylation.

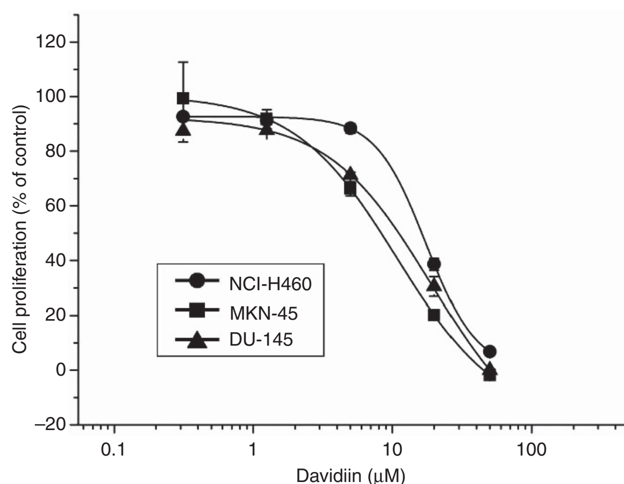

Figure 2 Davidiin inhibits cancer cells growth. Cells were treated with davidiin at various concentrations ranged from 0.3 to 50 μM for 96 h. Effects on cell proliferation were evaluated by WST-1 assays.

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