Chemistry & Biology
Brief Communication

Ginkgolic Acid Inhibits Protein SUMOylation by Blocking Formation of the E1-SUMO Intermediate

Isao Fukuda,[1,2] Akihiro Ito,[1,3,4,*] Go Hirai,[3,5] Shinichi Nishimura,[1] Hisashi Kawasaki,[6] Hisato Saitoh,[7] Ken-ichi Kimura,[8] Mikiko Sodeoka,[3,5] and Minoru Yoshida[1,2,3,4]

[1]Chemical Genetics Laboratory, RIKEN Advanced Science Institute, Wako, Saitama 351-0198, Japan
[2]Graduate School of Science and Engineering, Saitama University, 255 Shimo-okubo, Saitama, Saitama 338-8570, Japan
[3]Chemical Genomics Research Group, RIKEN Advanced Science Institute, Wako, Saitama 351-0198, Japan
[4]Japan Science and Technology Corporation, CREST Research Project, Kawaguchi, Saitama 332-0012, Japan
[5]Synthetic Organic Chemistry Laboratory, RIKEN Advanced Science Institute, Wako, Saitama 351-0198, Japan
[6]Department of Green and Sustainable Chemistry, Tokyo Denki University, 2-2 Nishiki-cho, Kanda, Chiyoda-ku, Tokyo 101-8457, Japan
[7]Department of Regulation Medicine, Institute of Molecular Embryology Genetics, Kumamoto University, Kumamoto 860-0811, Japan
[8]Laboratory of Chemical Biology, Department of Agro-Bioscience, Faculty of Agriculture, Iwate University, Morioka, Iwate 020-8550, Japan
*Correspondence: akihiro-i@riken.jp
DOI 10.1016/j.chembiol.2009.01.009

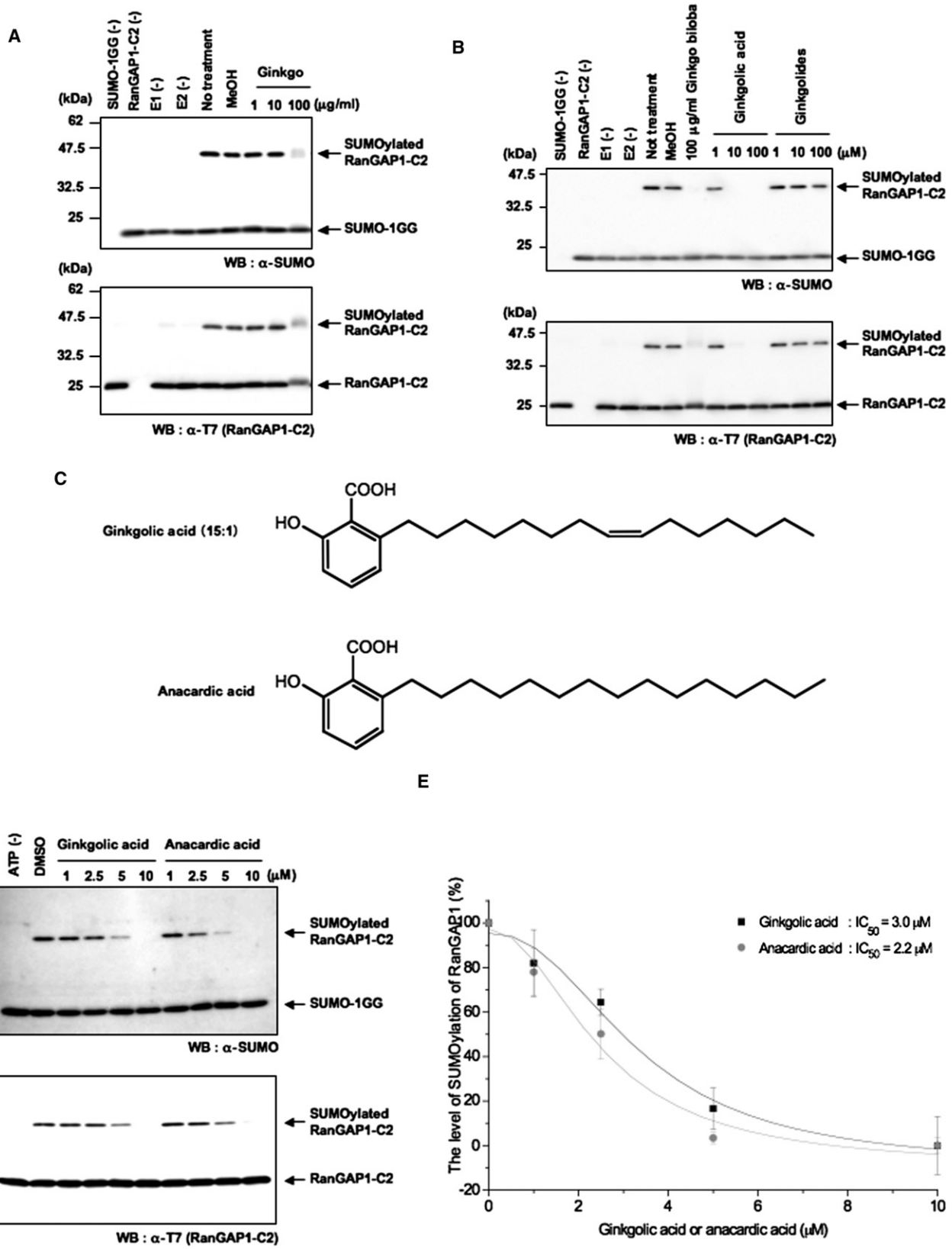
SUMMARY

Protein modification by small ubiquitin-related modifier proteins (SUMOs) controls diverse cellular functions. Dysregulation of SUMOylation or deSUMOylation processes has been implicated in the development of cancer and neurodegenerative diseases. However, no small-molecule inhibiting protein SUMOylation has been reported so far. Here, we report inhibition of SUMOylation by ginkgolic acid and its analog, anacardic acid. Ginkgolic acid and anacardic acid inhibit protein SUMOylation both in vitro and in vivo without affecting in vivo ubiquitination. Binding assays with a fluorescently labeled probe showed that ginkgolic acid directly binds E1 and inhibits the formation of the E1-SUMO intermediate. These studies will provide not only a useful tool for investigating the roles of SUMO conjugations in a variety of pathways in cells, but also a basis for the development of drugs targeted against diseases involving aberrant SUMOylation.

INTRODUCTION

Posttranslational modifications of proteins are the important mechanisms that regulate protein function, activity, or localization. These include phosphorylation, acetylation, methylation, and ubiquitination, which have been implicated in a variety of biological processes such as intercellular signaling, gene expression, and cell cycle control (Huang and Berger, 2008; Pickart, 2001; Yang and Seto, 2008). Perturbations within these modification systems have been shown to contribute to the etiology of various human diseases. Therefore, small-molecule compounds that modulate posttranslational modifications of proteins are thought to have potential to regulate biological processes and diseases. In recent years, posttranslational conjugation of small ubiquitin-related modifier protein (SUMO) to a specific lysine residue in a protein target has been shown as one of the major protein modifications that regulate various biological systems. Although SUMO and ubiquitin share structural similarities, their functional roles in cells are quite different. Conjugation of SUMO has been shown to alter diverse protein functions through changes in activity, subcellular localization, or stability, and it is thus involved in the regulation of many cellular pathways including transcription, intracellular transport, DNA repair, replication, and cell signaling (Johnson, 2004). SUMO modification has also been involved in tumorigenesis (Alarcon-Vargas and Ronai, 2002) and neurodegeneration (Dorval and Fraser, 2007), suggesting that SUMO modification is an important target for the development of drugs against these diseases.

SUMOylation is mediated by an enzymatic cascade reaction similar to ubiquitination (Johnson, 2004). In the first step, the SUMO precursor is processed by SUMO proteases to expose the C-terminal diglycine, which can then form a thioester bond with a cystein residue in the SUMO-activating enzyme (E1), the Aos1/Uba2 heterodimer, in an ATP-dependent manner. In the second step of the reaction, SUMO is transferred from E1 to a cystein residue in the SUMO-conjugating enzyme (E2), Ubc9, through another thioester bond. In the last step, SUMO forms an isopeptide bond with the ε-amino group of the target lysine residue through the function of E2 and the SUMO ligase (E3). Whereas E1 and E2 are sufficient for the in vitro SUMOylation of various substrates, several E3s facilitate both in vivo and in vitro conjugation (Johnson, 2004). Three types of E3 enzymes, PIAS, RanBP2, and PC2, have been described, all of which interact with Ubc9 and enhance both in vivo and in vitro SUMOylation (Johnson, 2004). SUMO can be deconjugated by isopeptidases, which cause removal of SUMO from its substrate (Johnson, 2004). These enzyme reactions are the potential targets for small molecules that control SUMOylation. Therefore, an in situ SUMOylation assay and a chemoluminescence-based assay for detecting SUMOylation have been developed and can be used for high-throughput screening for inhibitors of SUMOylation (Saitoh et al., 2006; Rouleau et al., 2008). Several chemical inhibitors of the ubiqutin E1 enzyme have been recently described (Sekizawa et al., 2002; Tsukamoto et al., 2005; Yang et al., 2007). In the case of SUMOylation, however, no chemical inhibitors have been reported so far.


A
SUMO-1GG (-)
RanGAP1-C2 (-)
E1 (-)
E2 (-)
No treatment
MeOH
Ginkgo
1 10 100 (μg/ml)
(kDa)
62
47.5
32.5
25
SUMOylated RanGAP1-C2
SUMO-1GG
WB : α-SUMO
RanGAP1-C2
WB : α-T7 (RanGAP1-C2)
B
Not treatment
100 μg/ml Ginkgo biloba
Ginkgolic acid
Ginkgolides
1 10 100 1 10 100 (μM)
C
Ginkgolic acid (15:1)
COOH
HO
Anacardic acid
D
ATP (-)
DMSO
Ginkgolic acid
Anacardic acid
1 2.5 5 10 1 2.5 5 10 (μM)
E
The level of SUMOylation of RanGAP1 (%)
Ginkgolic acid : IC50 = 3.0 μM
Anacardic acid : IC50 = 2.2 μM
Ginkgolic acid or anacardic acid (μM)

In this study, we screened for inhibitors of protein SUMOylation from a botanical extract library by using an in situ SUMOylation screening system. We found inhibitory activity of protein SUMOylation in the extract of *Ginkgo biloba* leaves and identified ginkgolic acid as an inhibitor. Ginkgolic acid and its structural analog anacardic acid inhibited both in vitro and in vivo SUMOylation, but not in vivo ubiquitination. Ginkgolic acid directly bound E1 and impaired the formation of the E1-SUMO intermediate. Discovery of the low-molecular inhibitor of protein SUMOylation will provide useful information about the enzymatic mechanism and drug development.

RESULTS AND DISCUSSION

Using an in situ cell-based SUMOylation assay method (Saitoh et al., 2006), we screened 500 samples of botanical extracts including food ingredients and found inhibitory activities of protein SUMOylation in 2 samples, including extract of *Gingko biloba* leaves. An in vitro SUMOylation assay with RanGAP1-C2, a C-terminal fragment of RanGAP1, as a substrate revealed that the extract of *Gingko biloba* leaves inhibited protein SUMOylation at the concentration of 100 μg/ml (Figure 1A). As ginkgolic acid and ginkgolides are two major components of the extract of *Gingko biloba* leaves, we first tested the effect of these compounds on the in vitro inhibition of protein SUMOylation; ginkgolic acid (Figure 1C), but not ginkgolides, completely inhibited the SUMOylation of RanGAP1-C2 in vitro at 10 μM (Figure 1B). Ginkgolic acid is an alkylphenol derivative that causes allergic skin inflammation. In addition to ginkgolic acid, anacardic acid (Figure 1C), a structurally related analog of ginkgolic acid known to be a histone acetyltransferase (HAT) inhibitor (Balasubramanyam et al., 2003), also inhibited the in vitro SUMOylation of RanGAP1-C2 (Figure 1D). IC_{50} values of ginkgolic acid and anacardic acid against the SUMOylation of RanGAP1-C2 are 3.0 μM and 2.2 μM, respectively (Figure 1E). We then asked whether ginkgolic acid and anacardic acid also inhibit in vivo protein SUMOylayion by analyzing the effect of the level of protein SUMOylation in 293T cells expressing Flag-tagged SUMO (Figure 2A). Immunoblotting with an anti-Flag antibody showed that ginkgolic acid and anacardic acid reduced the amount of high-molecular weight SUMO conjugates in a dose-dependent manner. Treatment with hydrogen peroxide also reduced the level of high-molecular weight SUMO conjugates (Figure 2A; see Figure S1 available online), as recently reported (Bossis and Melchior, 2006). Time course experiments revealed that inhibition of in vivo SUMOylation by ginkgolic acid or anacardic acid can be detected as early as 1 hr after the challenge (Figure S1). We next examined whether ginkgolic acid can inhibit the SUMOylation of p53, as p53 can be modified by SUMO in vivo on lysine residue 386 (Gostissa et al., 1999; Rodriguez et al., 1999) (Figure 2B). The level of SUMOylated p53 was markedly reduced by the ginkgolic acid treatment. Importantly, neither ginkgolic acid nor anacardic acid affected protein ubiquitination in cells (Figure 2C; Figure S1).

Ginkgolic acid is a structurally simple compound consisting of salicylic acid and a long-carbon chain substituent. We next examined which part is important for its inhibitory activity. Salicylic acid (Figure 3A) alone did not affect the in vitro SUMOylation of RanGAP1-C2 (Figure 3B) or the in vivo SUMOylation of p53 (Figure 2B), suggesting that the long carbon chain is necessary for its activity. However, because of the comparable or even stronger activity of anacardic acid, we speculated that the double bond within the alkyl chain may not be important. The role of two functional groups in ginkgolic acid, carboxylic acid and a hydroxyl group, were also examined by testing the activity of a ginkgolic acid methyl ester (Me-GA, Figure 3A) and an acetylated derivative of the phenolic hydroxyl group on ginkgolic acid (Ac-GA, Figure 3A; Figure 3C). Me-GA could not inhibit the SUMOylation of RanGAP1-C2 even at 10 μM, whereas Ac-GA retained the ability to inhibit the SUMOylation of RanGAP1-C2. These results suggest that the carboxylic acid in ginkgolic acid is indispensable for the inhibitory activity of ginkgolic acid.

To elucidate the mechanism by which ginkgolic acid inhibits protein SUMOylation, we synthesized a derivative with the fluorescent substance BODIPY (GA-BODIPY, Figure 4A) as a probe to investigate the target of ginkgolic acid. Before the detailed analysis, we confirmed that GA-BODIPY, but not BODIPY alone (C5-BODIPY, Figure 4A), effectively inhibited the in vitro SUMOylation of RanGAP1-C2 (Figure S2). When GA-BODIPY was added to the complete reaction mixture of in vitro SUMOylation, we observed dose-dependent binding of E1 to GA-BODIPY, but not C5-BODIPY, in gel electrophoresis in both nondenatured conditions (Figure 4B) and denatured conditions (Figure S3), suggesting that E1 is the target of ginkgolic acid. To confirm this idea, we incubated GA-BODIPY with each recombinant protein: E1, E2, or GST alone. GA-BODIPY binds only to E1, but not to E2 or GST (Figure 4C). This binding to E1 is specific, because ginkgolic acid inhibited the binding between E1 and GA-BODIPY in a dose-dependent manner (Figure 4D). Importantly, however, inactive derivatives, salicylic acid and Me-GA, failed to inhibit the binding. In contrast, the active derivative Ac-GA could compete for binding (Figure 4D). These results suggest that E1 is the specific and direct target of ginkgolic acid, and that the long carbon chain and the carboxylic acid group of ginkgolic acid are essential for the interaction with E1.

Figure 1. Ginkgolic Acid, a Major Component of *Ginkgo biloba* Extract, Inhibited SUMOylation In Vitro

(A) Indicated concentrations of the extract of *Ginkgo biloba* leaves (1–100 μg/ml) 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), and His-tagged Ubc9 (E2) in the presence of 2 mM ATP. SUMOlylated RanGAP1-C2 was detected by immunoblotting with an anti-T7 or anti-SUMO-1 antibody.

(B) Indicated concentrations of ginkgolic acid or ginkgolides (1–100 μM) were added to the SUMOylation reaction mixture, and then SUMOlylated RanGAP1-C2 was detected as described in (A).

(C) Structure of ginkgolic acid, a major component of *Ginkgo biloba* extract, and its analog anacardic acid.

(D and E) Inhibition of in vitro SUMOylation by ginkgolic acid and anacardic acid. Ginkgolic acid or anacardic acid at indicated concentrations (1–10 μM) was added to the reaction mixture, and then SUMOlylated RanGAP1-C2 was detected as described in (A). The level of the SUMOylation of RanGAP1-C2 was determined by measuring the intensity of SUMOylated RanGAP1-C2 by 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 by using 50% inhibition compared with a control sample without compounds.

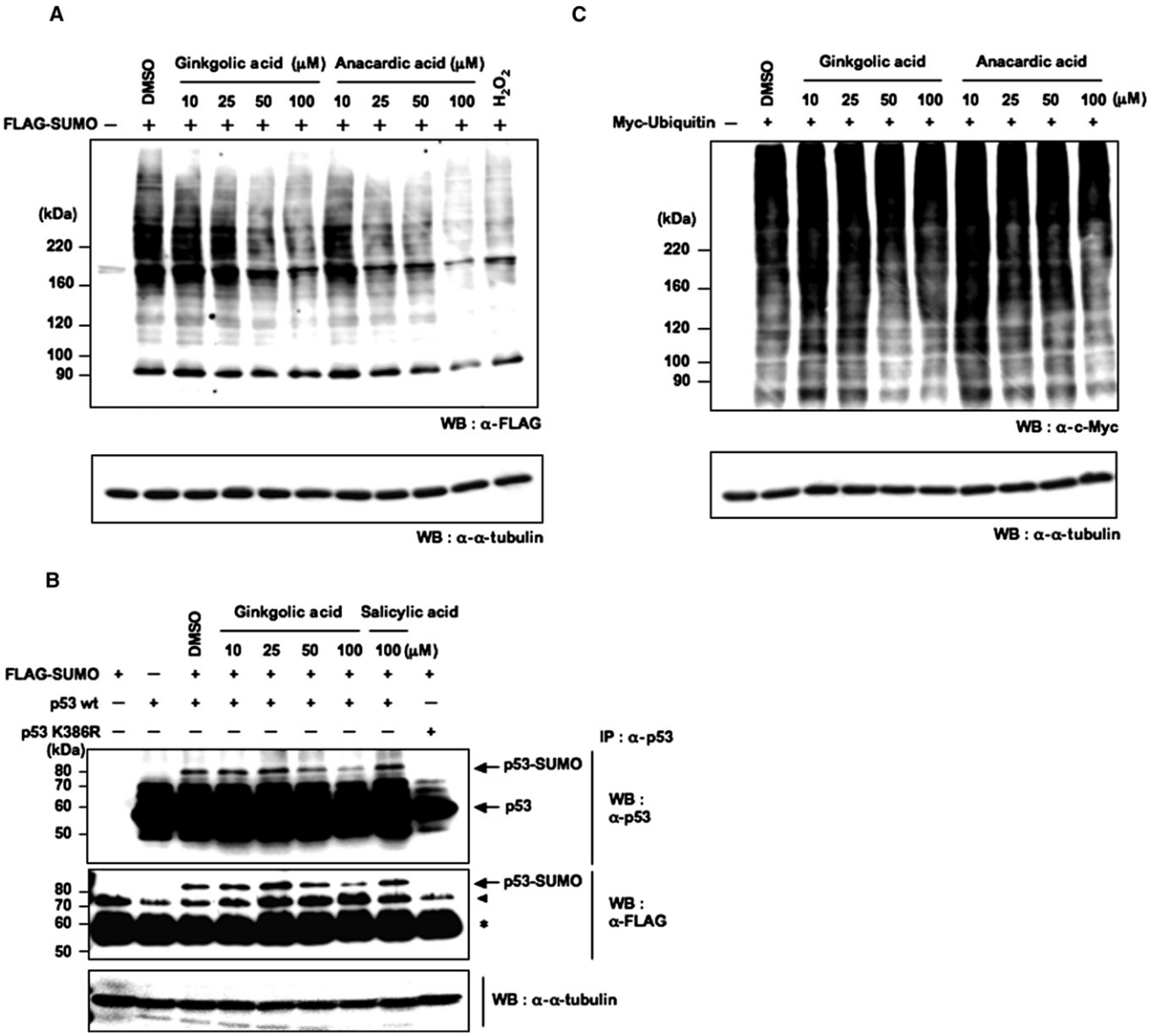


Figure 2. Ginkgolic Acid Inhibited SUMOylation In Vivo

(A) Inhibition of in vivo protein SUMOylation by ginkgolic acid and anacardic acid. 293T cells were transfected with Flag-tagged SUMO and then treated with various concentrations of ginkgolic acid or anacardic acid (10–100 μM) for 4 hr or were treated with 1 mM H_2O_2 for 1 hr. Cells were lysed in RIPA buffer containing 50 mM *N*-ethylmaleimide, and the lysates were separated by 6% SDS-PAGE, followed by immunoblotting with an anti-FLAG antibody.

(B) Inhibition of in vivo p53 SUMOylation by ginkgolic acid. H1299 cells ($p53^{-/-}$) that had been transfected with the indicated combinations of Flag-tagged SUMO, p53 wild-type, and SUMOylation-deficient mutant K386R were treated with various concentrations of ginkgolic acid, anacardic acid (10–100 μM), or 100 μM salicylic acid for 18 hr. Cell extracts were immunoprecipitated with an anti-p53 (FL393)-G antibody, followed by immunoblotting with an anti-FLAG or anti-p53 (Ab-6) antibody. The arrowhead indicates a nonspecific band, and the asterisk indicates antibody heavy chain.

(C) The effects of ginkgolic acid and anacardic acid on in vivo ubiquitination. 293T cells that had been transfected with Myc-tagged ubiquitin were treated with various concentrations of ginkgolic acid or anacardic acid (10–100 μM) for 4 hr in the presence of 10 μM of the proteasome inhibitor MG132 (in order to increase the level of ubiquitination by blocking the degradation of ubiquitinated protein). Cells were lysed in RIPA buffer containing 50 mM *N*-ethylmaleimide, and the lysates were separated by 6% SDS-PAGE, followed by immunoblotting with an anti-c-Myc antibody.

Finally, we sought to determine whether ginkgolic acid and anacardic acid could block the formation of the E1-SUMO-1 intermediate. The complex of E1 and SUMO-1 biotinylated via the thioester bond can be detected in the presence of ATP under nonreducing conditions by using a biotin-avidin detection system (Uchimura et al., 2004) (Figure 4E). The band corresponding to the E1-biotinylated SUMO-1 intermediate was detected after incubating biotinylated SUMO-1 with E1 in the presence of ATP, but this band disappeared after the addition of the reducing agent DTT. The formation of the E1-biotinylated SUMO-1 intermediate was blocked by ginkgolic acid and anacardic acid in a dose-dependent manner (Figure 4E). Thus, we conclude that ginkgolic

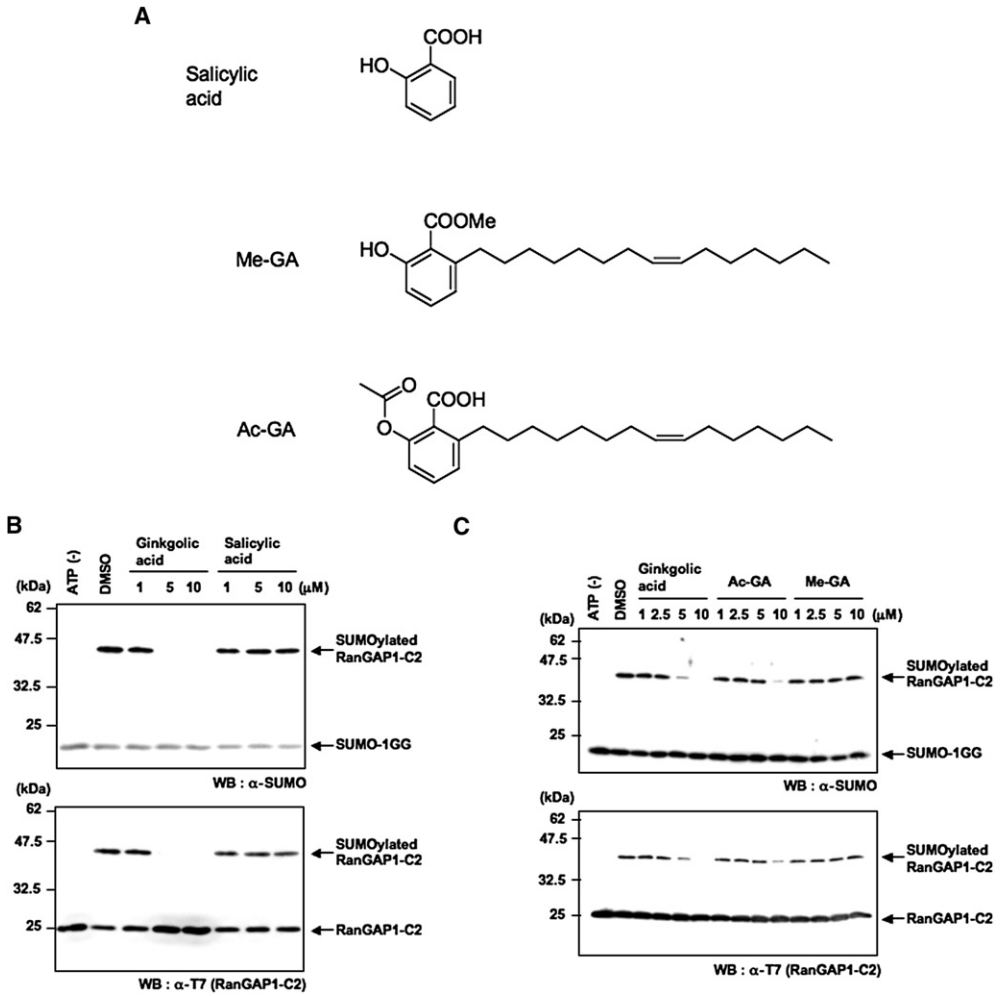


Figure 3. Structure-Activity Relationship of Ginkgolic Acid

(A) Structure of salicylic acid, a ginkgolic acid methyl ester (Me-GA), and an acetylated derivative of the phenolic hydroxyl group on ginkgolic acid (Ac-GA). (B and C) Effects of salicylic acid, Me-GA, or Ac-GA on in vitro SUMOylation. Various concentrations of the compounds (1–10 μM) were added to the SUMOylation reaction mixture, and then SUMOlylated RanGAP1-C2 was detected as described in Figure 1A.

acid inhibits protein SUMOylation by directly binding to E1 and thereby blocking the formation of the E1-SUMO-1 intermediate.

Both ginkgolic acid and anacardic acid possess diverse activities. Ginkgolic acid induces neuronal cell death and activates protein phosphatase 2C (PP2C) (Ahlemeyer et al., 2001). However, anacardic acid inhibits the activities of diverse enzymes, including lipoxygenase (Grazzini et al., 1991) and HATs (Balasubramanyam et al., 2003), and activates Aurora kinase A (Kishore et al., 2008). Therefore, it appears that inhibition of protein SUMOylation is not the only activity of ginkgolic acid and anacardic acid. Indeed, both ginkgolic acid and anacardic acid inhibited the PCAF-mediated acetylation of histones in vitro at a concentration of 10 μM, as previously reported (Figure S4) (Balasubramanyam et al., 2003). The effective concentration was similar to that for inhibition of the SUMOylation of RanGAP1-C2 in vitro (Figure 1). However, both compounds could not affect the acetylation of histones in cells even at the concentration of 100 μM (Figure S4), a concentration that is sufficient to inhibit in vivo protein SUMOylation (Figures 2A and 2B; Figure S1). Furthermore, more than 100 μM ginkgolic acid is necessary for activating PP2C in vitro (Ahlemeyer et al., 2001). Although anacardic acid inhibited lipoxygenase and activated Aurora kinase A in vitro at the concentration of ~10 μM (Grazzini et al., 1991; Kishore et al., 2008), it is unclear whether anacardic acid could affect the in vivo activity of these enzymes. These observations suggest that SUMOylation is one of most sensitive enzyme reactions targeted by ginkgolic acid and anacardic acid. Anacardic acid also exhibits antitumor activity (Rea et al., 2003) and sensitizes tumor cells to ionizing radiation through inhibition of nuclear factor-κB (NF-κB) signaling pathways (Sung et al., 2008), although its molecular mechanism is not yet fully understood. As SUMO conjugation plays an important role in the regulation of NF-κB signaling pathways (Mabb and Miyamoto, 2007), it seems possible that inhibition of the NF-κB signaling pathways by anacardic acid is mediated by suppression of the SUMOylation of proteins regulating NF-κB activity such as IκB or NEMO. An active derivative, Ac-GA, inhibited not only SUMOylation, but also the proliferation of cancer cells, whereas the inactive

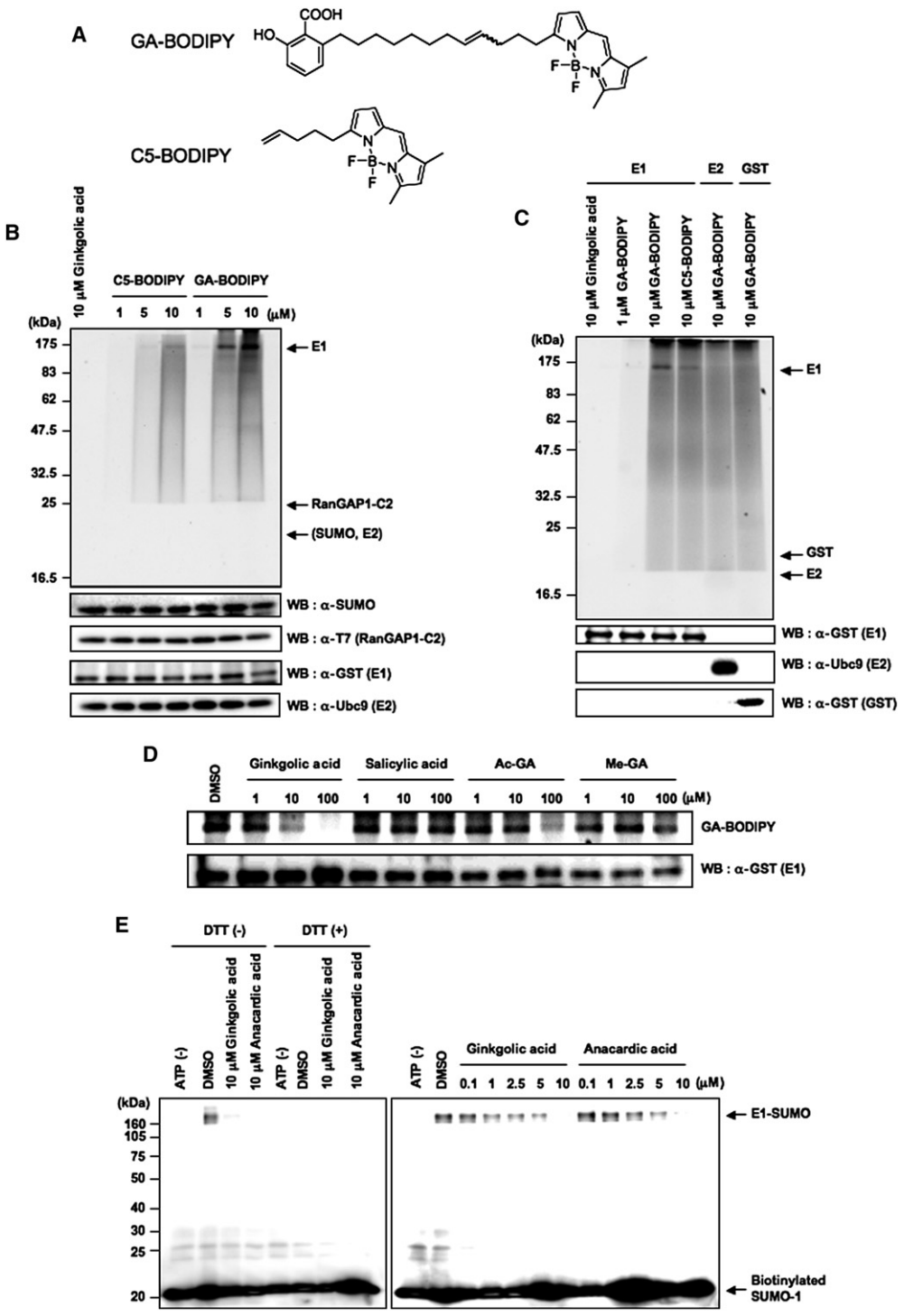


Figure 4. E1 Is the Target of Ginkgolic Acid

(A) Structure of a derivative with fluorescent substance BODIPY (GA-BODIPY) and BODIPY alone (C5-BODIPY).

(B and C) Specific binding of GA-BODIPY to E1. GA-BODIPY, C5-BODIPY, or ginkgolic acid at various concentrations (1–10 μM) was incubated with the SUMOylation reaction mixture containing (B) His-tagged SUMO-1, His- and T7-tagged RanGAP1-C2, GST-Aos1/Uba2, and His-tagged Ubc9 or with either (C) GST-Aos1/Uba2, His-tagged Ubc9, or GST alone in the presence of ATP. The proteins were separated by SDS-PAGE under nondenaturing conditions (without 2-mercaptoethanol and boiling). The bound protein was detected by using a fluorescence detector with excitation at 488 nm. The amount of each protein was assessed by SDS-PAGE under the same nondenaturing conditions, followed by immunoblotting with anti-SUMO (SUMO), anti-T7 (RanGAP1-C2), anti-GST (E1), and anti-Ubc9 (E2) antibodies, respectively. The molecular sizes of each protein detected by immunoblotting were indicated by arrows. The end of nonspecific background labeling around 25 kDa appeared reproducibly by an unknown reason, although the gel was run to the bottom.

derivative, Me-GA, did not (Figure S5), suggesting a link between SUMOylation inhibition and cytotoxicity. However, it is still unclear whether the various pharmacological activities of these compounds can be ascribed to inhibition of SUMOylation.

In this study, we show that both the carboxylic group and the long aliphatic chain are important for inhibition of SUMOylation by binding to E1. One could speculate that the carboxyl group of ginkgolic acid, like SUMO, forms a thioester bond with the sulfhydryl group of the active site cysteine of E1 to inhibit the formation of the E1-SUMO intermediate. However, this possibility was ruled out, because GA-BODIPY effectively bound to the inactive E1 C173S mutant, in which the active site cysteine residue is mutated to serine (Figure S6). The molecular mechanism by which ginkgolic acid binds and inhibits E1 is an important issue that should be elucidated in the future. Additional information about the structure-activity relationship will be useful for analyzing the mode of inhibition and also in the design of a novel SUMOylation inhibitor that lacks the undesirable activities of ginkgolic acid; such a compound would be more suitable as a lead compound for drug development.

SIGNIFICANCE

The posttranslational modification by SUMO has emerged as a central regulatory mechanism of protein function, and it may be implicated in several diseases. In this study, using an in situ cell-based screening system to select compounds from botanical extracts libraries, we identified ginkgolic acid and anacardic acid as small-molecule inhibitors of protein SUMOylation. Mechanistically, ginkgolic acid impaired SUMOylation by blocking the formation of an E1-SUMO thioester complex, by directly binding to E1. Structure-function analysis demonstrated that both the carboxylic acid group and the long aliphatic chain are essential for binding to E1 and for inhibition of SUMOylation. Although a variety of in vivo activities of ginkgolic acid and anacardic acid have been reported, it is currently unclear whether their SUMOylation inhibition is responsible for these activities. Elucidation of the molecular mechanism by which these compounds inhibit E1 activity will provide a basis for the design and development of novel SUMOylation inhibitors.

EXPERIMENTAL PROCEDURES

Experimental Procedures are described in Supplemental Data.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, Supplemental References, and six figures and can be found with this article online at http://www.cell.com/chemistry-biology/supplemental/S1074-5521(09)00034-9.

ACKNOWLEDGMENTS

We thank Y. Uchimura (Kumamoto University) for a technical suggestion. This work was supported in part by the Chemical Genomics Research Group Project, RIKEN Advanced Science Institute, the CREST Research Project, the Japan Science and Technology Corporation, and a Grant-in-Aid for Scientific Research on Priority Area "Cancer" from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Received: November 9, 2008
Revised: January 10, 2009
Accepted: January 14, 2009
Published: February 26, 2009

REFERENCES

Ahlemeyer, B., Selke, D., Schaper, C., Klumpp, S., and Krieglstein, J. (2001). Ginkgolic acids induce neuronal death and activate protein phosphatase type-2C. Eur. J. Pharmacol. *430*, 1–7.

Alarcon-Vargas, D., and Ronai, Z. (2002). SUMO in cancer–wrestlers wanted. Cancer Biol. Ther. *1*, 237–242.

Balasubramanyam, K., Swaminathan, V., Ranganathan, A., and Kundu, T.K. (2003). Small molecule modulators of histone acetyltransferase p300. J. Biol. Chem. *278*, 19134–19140.

Bossis, G., and Melchior, F. (2006). Regulation of SUMOylation by reversible oxidation of SUMO conjugating enzymes. Mol. Cell *21*, 349–357.

Dorval, V., and Fraser, P.E. (2007). SUMO on the road to neurodegeneration. Biochim. Biophys. Acta *1773*, 694–706.

Gostissa, M., Hengstermann, A., Fogal, V., Sandy, P., Schwarz, S.E., Scheffner, M., and Del Sal, G. (1999). Activation of p53 by conjugation to the ubiquitin-like protein SUMO-1. EMBO J. *18*, 6462–6471.

Grazzini, R., Hesk, D., Heininger, E., Hildenbrandt, G., Reddy, C.C., Cox-Foster, D., Medford, J., Craig, R., and Mumma, R.O. (1991). Inhibition of lipoxygenase and prostaglandin endoperoxide synthase by anacardic acids. Biochem. Biophys. Res. Commun. *176*, 775–780.

Huang, J., and Berger, S.L. (2008). The emerging field of dynamic lysine methylation of non-histone proteins. Curr. Opin. Genet. Dev. *18*, 152–158.

Johnson, E.S. (2004). Protein modification by SUMO. Annu. Rev. Biochem. *73*, 355–382.

Kishore, A.H., Vedamurthy, B.M., Mantelingu, K., Agrawal, S., Reddy, B.A., Roy, S., Rangappa, K.S., and Kundu, T.K. (2008). Specific small-molecule activator of Aurora kinase A induces autophosphorylation in a cell-free system. J. Med. Chem. *51*, 792–797.

Mabb, A.M., and Miyamoto, S. (2007). SUMO and NF-κB ties. Cell. Mol. Life Sci. *64*, 1979–1996.

Pickart, C.M. (2001). Mechanisms underlying ubiquitination. Annu. Rev. Biochem. *70*, 503–533.

Rea, A.I., Schmidt, J.M., Setzer, W.N., Sibanda, S., Taylor, C., and Gwebu, E.T. (2003). Cytotoxic activity of *Ozoroa insignis* from Zimbabwe. Fitoterapia *74*, 732–735.

Rodriguez, M.S., Desterro, J.M., Lain, S., Midgley, C.A., Lane, D.P., and Hay, R.T. (1999). SUMO-1 modification activates the transcriptional response of p53. EMBO J. *18*, 6455–6461.

Rouleau, N., Wang, J., Karras, L., Andrews, E., Bielefeld-Sevigny, M., and Chen, Y. (2008). Highly sensitive assays for SUMOylation and small ubiquitin-like modifier-dependent protein-protein interactions. Anal. Biochem. *375*, 364–366.

(D) Competition assay. Ginkgolic acid, salicylic acid, Ac-GA, or Me-GA at indicated concentrations was added with 10 μM GA-BODIPY to GST-Aos1/Uba2 (E1), and the binding of GA-BODIPY to E1 was detected as described above. The amount of GST-E1 was assessed by immunoblotting with an anti-GST antibody. (E) Impairment of the thioester bond formation between E1 and biotinylated SUMO-1 by ginkgolic acid or anacardic acid. Ginkgolic acid or anacardic acid at 10 μM (left panel) or various concentrations (0.1–10 μM; right panel) was added to a reaction mixture containing 0.1 μg biotinylated SUMO-1 and 1 μg 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 with 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 (left panel).

Saitoh, N., Uchimura, Y., Tachibana, T., Sugahara, S., Saitoh, H., and Nakao, M. (2006). In situ SUMOylation analysis reveals a modulatory role of RanBP2 in the nuclear rim and PML bodies. Exp. Cell Res. *312*, 1418–1430.

Sekizawa, R., Ikeno, S., Nakamura, H., Naganawa, H., Matsui, S., Iinuma, H., and Takeuchi, T. (2002). Panepophenanthrin, from a mushroom strain, a novel inhibitor of the ubiquitin-activating enzyme. J. Nat. Prod. *65*, 1491–1493.

Sung, B., Pandey, M.K., Ahn, K.S., Yi, T., Chaturvedi, M.M., Liu, M., and Aggarwal, B.B. (2008). Anacardic acid (6-nonadecyl salicylic acid), an inhibitor of histone acetyltransferase, suppresses expression of nuclear factor-κB-regulated gene products involved in cell survival, proliferation, invasion, and inflammation through inhibition of the inhibitory subunit of nuclear factor-κBα kinase, leading to potentiation of apoptosis. Blood *111*, 4880–4891.

Tsukamoto, S., Hirota, H., Imachi, M., Fujimuro, M., Onuki, H., Ohta, T., and Yokosawa, H. (2005). Himeic acid A: a new ubiquitin-activating enzyme inhibitor isolated from a marine-derived fungus, *Aspergillus* sp. Bioorg. Med. Chem. Lett. *15*, 191–194.

Uchimura, Y., Nakao, M., and Saitoh, H. (2004). Generation of SUMO-1 modified proteins in *E. coli*: towards understanding the biochemistry/structural biology of the SUMO-1 pathway. FEBS Lett. *564*, 85–90.

Yang, X.J., and Seto, E. (2008). Lysine acetylation: codified crosstalk with other posttranslational modifications. Mol. Cell *31*, 449–461.

Yang, Y., Kitagaki, J., Dai, R.M., Tsai, Y.C., Lorick, K.L., Ludwig, R.L., Pierre, S.A., Jensen, J.P., Davydov, I.V., Oberoi, P., et al. (2007). Inhibitors of ubiquitin-activating enzyme (E1), a new class of potential cancer therapeutics. Cancer Res. *67*, 9472–9481.

