Ginkgolic acid inhibits protein SUMOylation by blocking formation of the E1-SUMO intermediate

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Running title: Ginkgolic acid as an inhibitor of SUMOylation

Summary

Protein modification by small ubiquitin-like modifiers (SUMO) 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 analogue, anacardic acid. Ginkgolic acid and anacardic acid inhibit protein SUMOylation both *in vitro* and *in vivo* without affecting *in vivo* ubiquitination. Binding assays using 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 is thus involved in 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 development of drugs against these diseases.

The SUMOylation is mediated by an enzymatic cascade reaction similar to ubqutination (Johnson, 2004). At the first step, the SUMO precursor is processed by SUMO proteases to expose the C-terminal diglycine, which then can 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). While E1 and E2 are sufficient for *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, which 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.

In this study, we screened for inhibitors of protein SUMOylation from a botanical extract library 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 E1-SUMO intermediate. Discovery of the low molecular inhibitor of protein SUMOlylation 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 two samples, including extract of *Gingko biloba* leaves. *In vitro* SUMOylation assay using 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 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 acetyltransferases (HATs) inhibitor (Balasubramanyam et al., 2003), also inhibited *in vitro* SUMOylation of RanGAP1-C2 (Figure 1D). IC_{50} values of ginkgolic acid and anacardic acid against 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 using 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 and Figure S1) 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 h after the challenge (Figure S1). We next examined whether ginkgolic acid can inhibit 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 and 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 *in vitro* SUMOylation of RanGAP1-C2 (Figure 3B) and *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 SUMOylation of RanGAP1-C2 even at 10 μM, whereas Ac-GA retained the ability to inhibit 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 *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 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 the long carbon chain and the carboxylic acid group of ginkgolic acid are essential for the interaction with E1.

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 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 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 acid inhibits protein SUMOylation by directly binding to E1 and thereby blocking formation of the

E1-SUMO-1 intermediate.

Both ginkgolic acid and anacardic acid possess diverse activities. Ginkgolic acid induces neuronal cell death and activate protein phosphatase 2C (PP2C) (Ahlemeyer et al., 2001). On the other hand, anacardic acid inhibits activities of diverse enzymes including lipoxygenase (Grazzini et al., 1991) and histone acetyltransferases (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 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 SUMOylation of RanGAP1-C2 *in vitro* (Figure 1). However, both compounds could not affect 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 (Figure 2A, B and Figure S1). Furthermore, more than 100 μM of 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 around 10 μM (Grazzini et al., 1991; Kishore et al., 2008), it is unclear whether anacardic acid could affect *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 anti-tumor 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 has not been 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 SUMOylation of proteins regulating NF–κB activity such as IκB or NEMO. An active derivative, Ac-GA, inhibited not only SUMOylation but also proliferation of cancer cells, whereas the inactive derivative Me-GA did not (Figure S5), suggesting a link between the 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 showed 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. Further information about the structure-activity relationship will be useful for analyzing the mode of inhibition and also in the design of 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 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 in are essential for binding to E1 and 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

Methods

A detailed description of methods used for protein expression and purification, cell culture, transfection, immunoblotting, immunoprecipitation, *in vitro* SUMOylation assay, assay for SUMO-1 thioester bond formation, GA-BODIPY-binding assay, *in vitro* HAT assay, WST-1 assay, chemical synthesis are described in supplemental data.

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Figure Legends

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), His-tagged-Ubc9 (E2) in the presence of 2 mM ATP. SUMOlylated RanGAP1-C2 was detected by immunoblotting using 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 Fig. 1A. (**C**) Structure of ginkgolic acid, a major component of Ginkgo biloba extract, and its analogue anacardic acid. (**D, 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 Fig. 1A. The level of 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 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 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. (**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 of salicylic acid for 18 h. 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**) 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 h in the presence of 10 μM of the proteasome inhibitor MG132 (in order to increase the level of ubiquitination by blocking 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 using an anti-c-Myc antibody.

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, 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

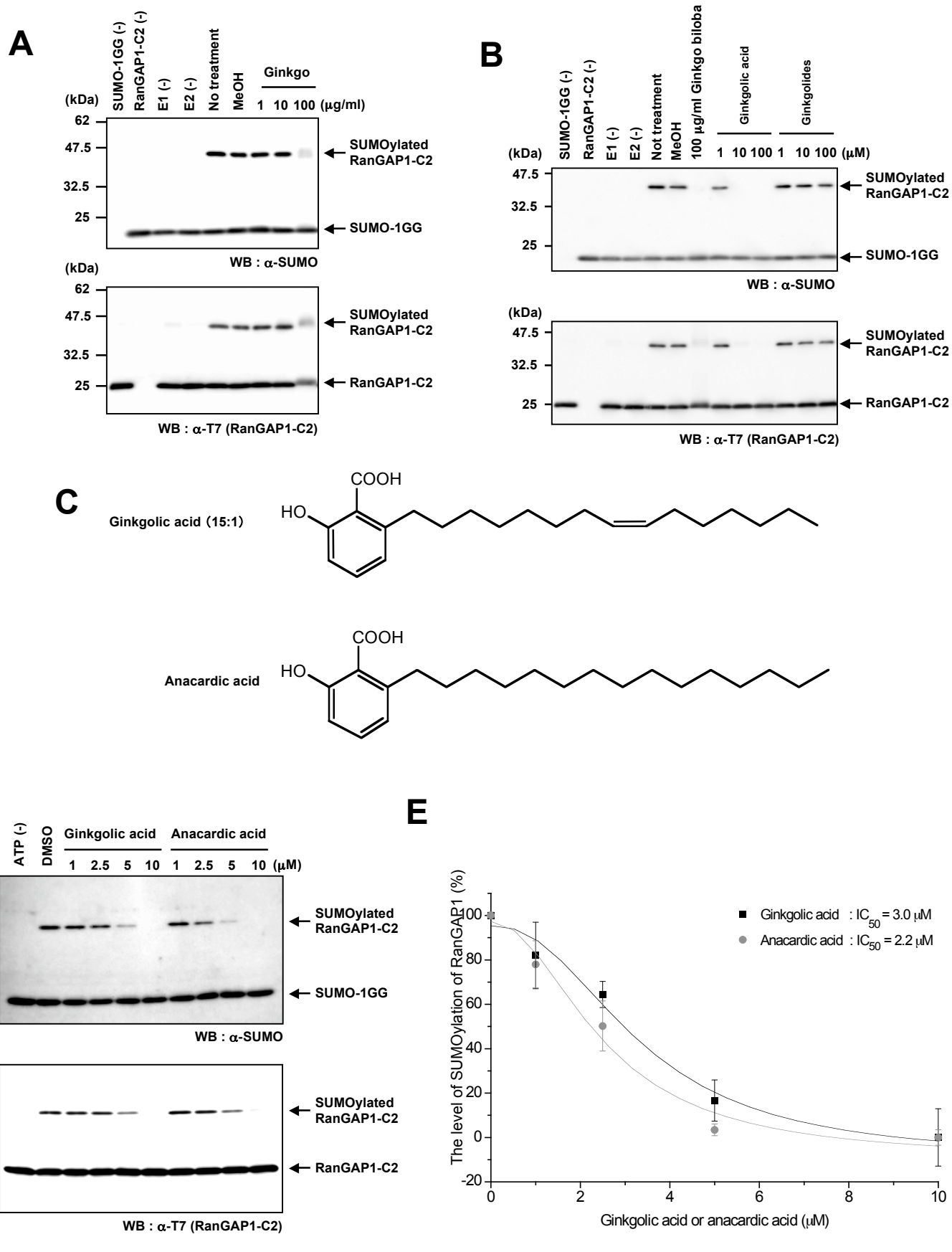
SUMOlylated RanGAP1-C2 was detected as described in Fig. 1A.

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, 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 His-tagged SUMO-1, His and T7-tagged RanGAP1-C2, GST-Aos1/Uba2, His-tagged Ubc9 (B) or with either GST-Aos1/Uba2, His-tagged Ubc9, or GST alone (C) 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 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. Molecular sizes of each protein detected by immunoblotting were indicated by arrows. The end of non-specific background labeling around 25 kDa appeared reproducibly by an unknown reason, although the gel was run to the bottom. (**D**) Competition assay. Ginkgolic acid, salicylic acid, Ac-GA, or Me-GA at indicated concentrations was added with 10 μM of 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 of biotinylated SUMO-1 and 1 μg 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 of DTT to the reaction completely abolished the complex formation of biotinylated SUMO-1 and GST-Aos1/Uba2 (left panel).

Figure 1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E1 (-)
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(kDa)
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32.5
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ATP (-)
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1 2.5 5 10 1 2.5 5 10 (μM)
E
The level of SUMOylation of RanGAP1 (%)
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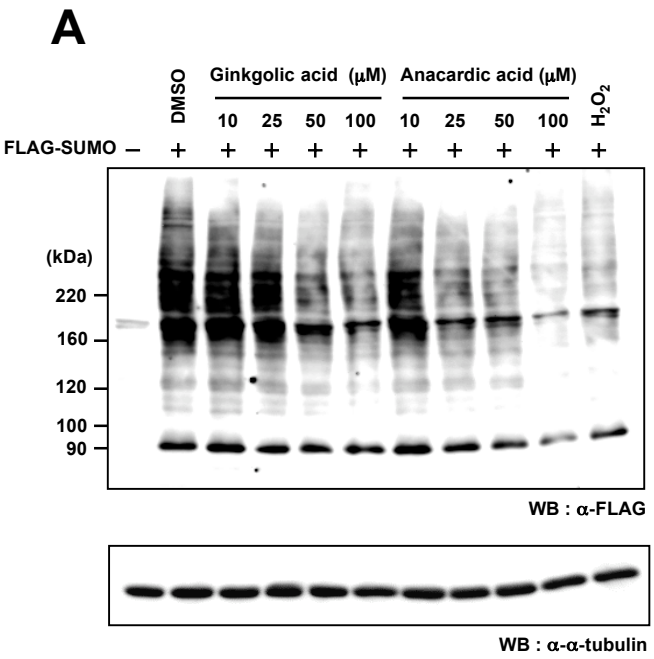
Anacardic acid : IC50 = 2.2 μM
Ginkgolic acid or anacardic acid (μM)

Figure 2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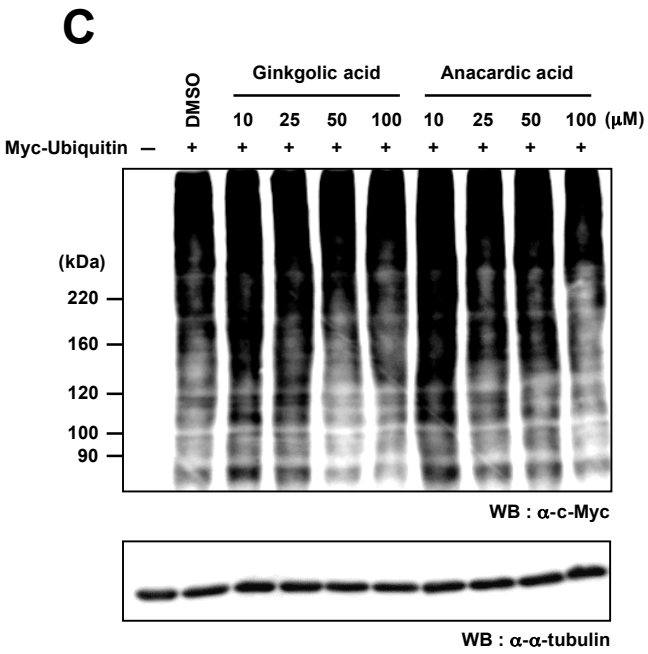
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90
WB : α-c-Myc
WB : α-α-tubulin

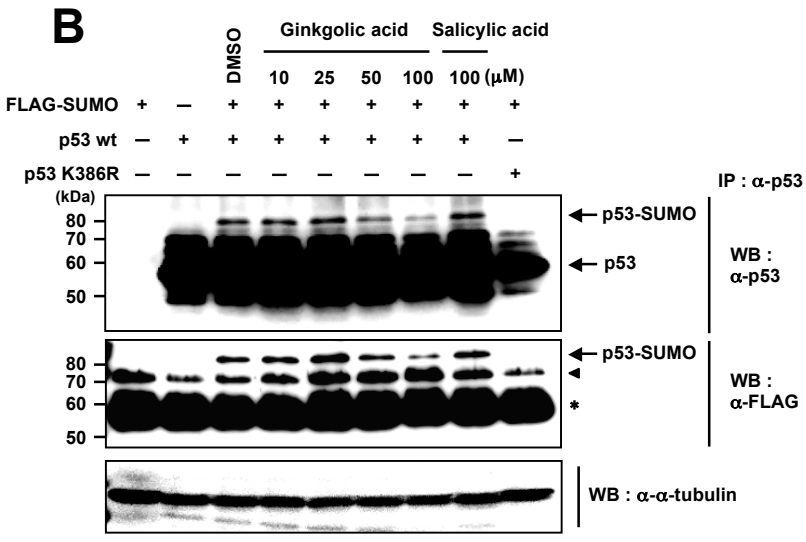
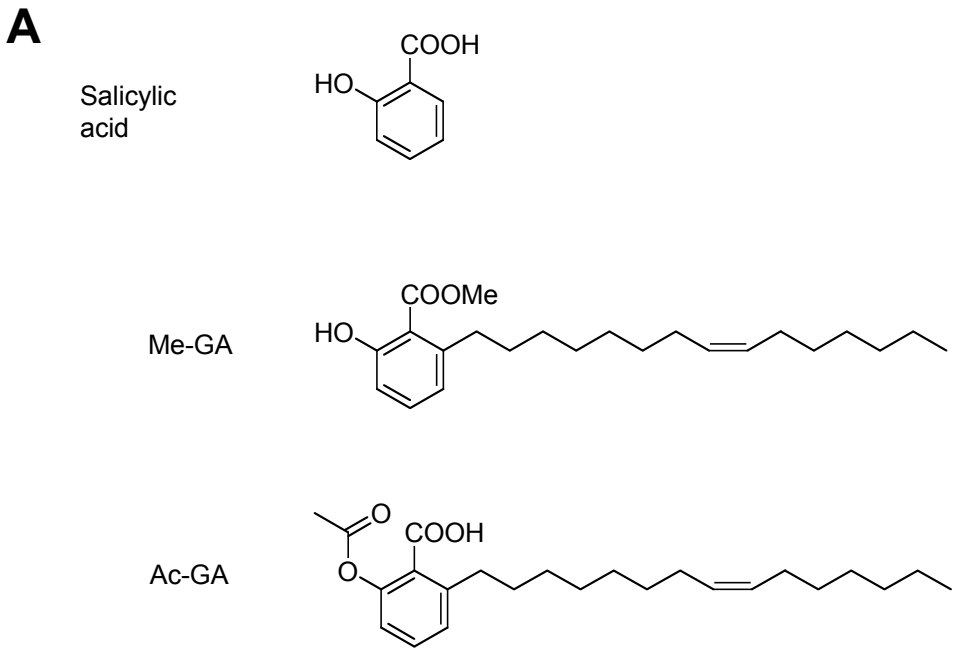

B
DMSO
Ginkgolic acid
Salicylic acid
10 25 50 100 100 (μM)
FLAG-SUMO + – + + + + + + +
p53 wt – + + + + + + + –
p53 K386R – – – – – – – – +
IP : α-p53
(kDa)
80
70
60
50
p53-SUMO
p53
WB : α-p53
80
70
60
50
p53-SUMO
*
WB : α-FLAG
WB : α-α-tubulin

Figure 3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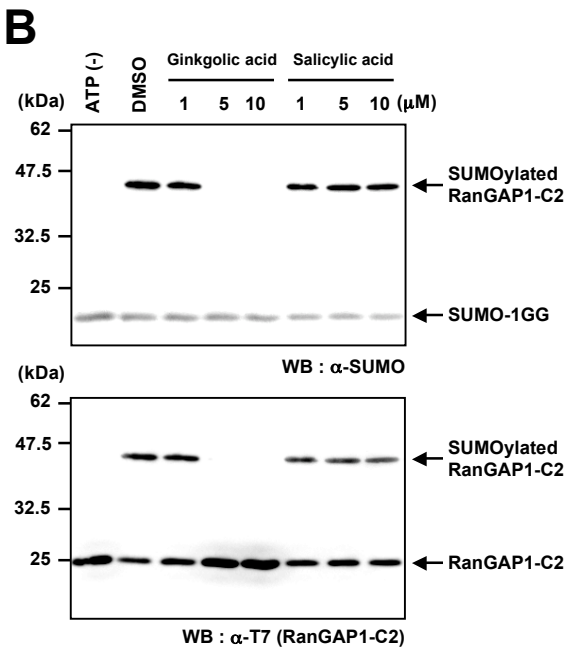
Salicylic acid
COOH
HO
Me-GA
COOMe
HO
Ac-GA
O
O
COOH


B
ATP (-)
DMSO
Ginkgolic acid
Salicylic acid
(kDa)
1 5 10 1 5 10 (μM)
62
47.5
32.5
25
SUMOylated RanGAP1-C2
SUMO-1GG
WB : α-SUMO
(kDa)
62
47.5
32.5
25
SUMOylated RanGAP1-C2
RanGAP1-C2
WB : α-T7 (RanGAP1-C2)

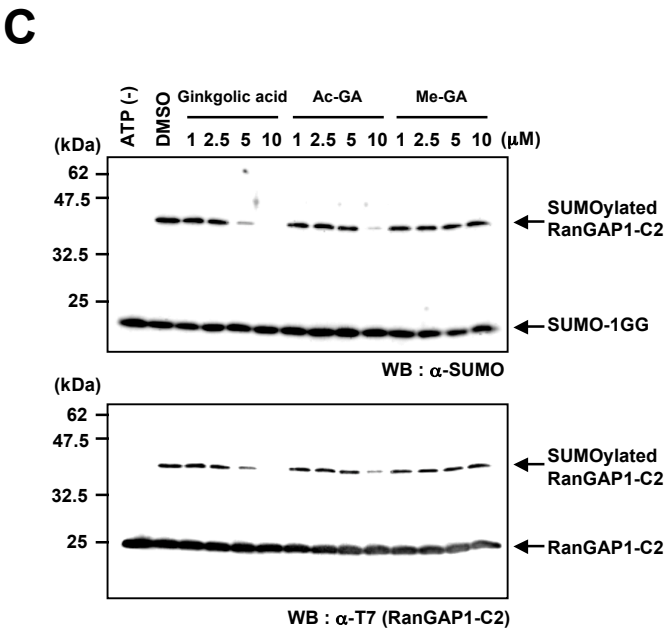
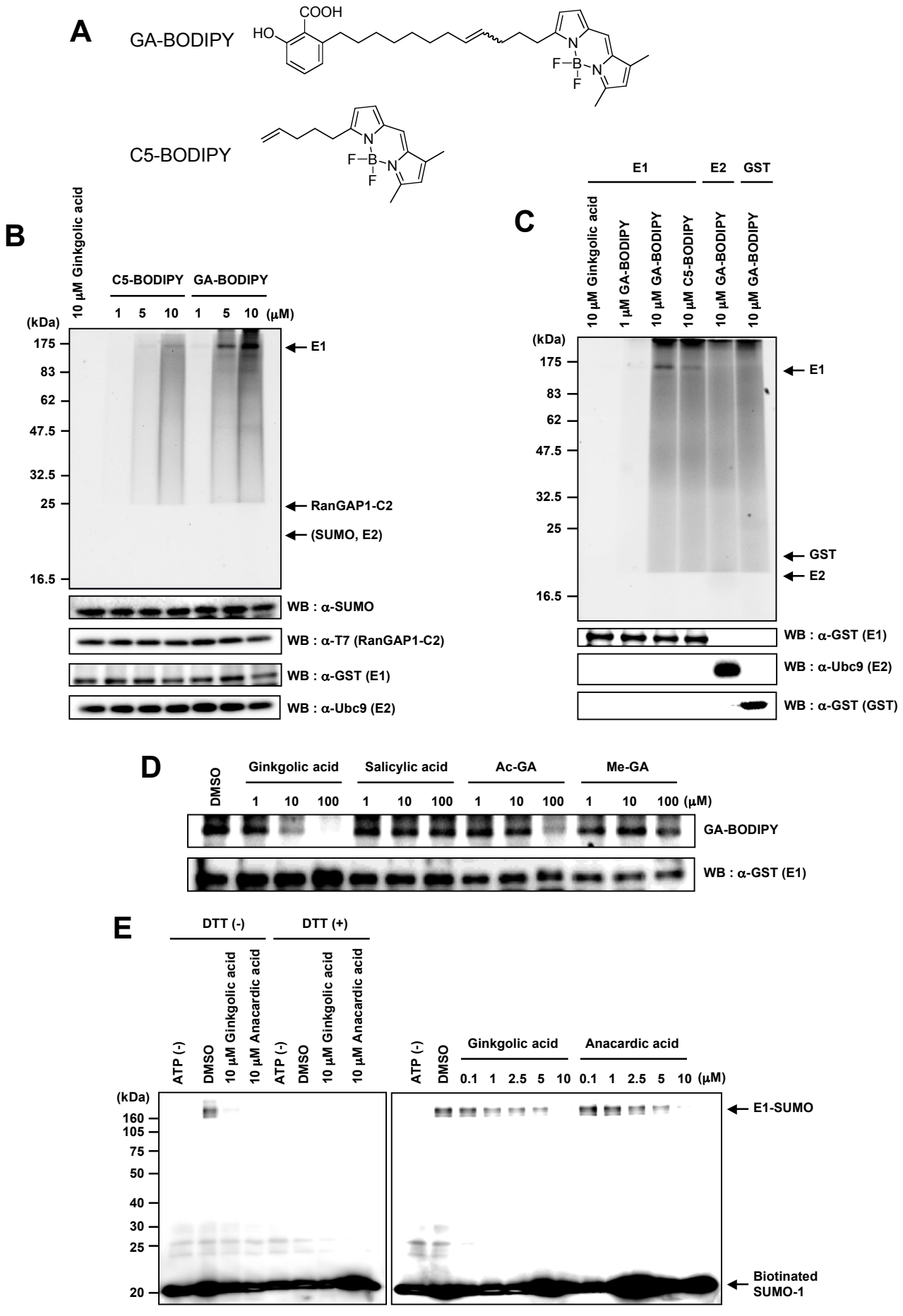

C
ATP (-)
DMSO
Ginkgolic acid
Ac-GA
Me-GA
(kDa)
1 2.5 5 10 1 2.5 5 10 1 2.5 5 10 (μM)
62
47.5
32.5
25
SUMOylated RanGAP1-C2
SUMO-1GG
WB : α-SUMO
(kDa)
62
47.5
32.5
25
SUMOylated RanGAP1-C2
RanGAP1-C2
WB : α-T7 (RanGAP1-C2)

Figure 4


A
GA-BODIPY
COOH
HO
N
F–B–N
F
C5-BODIPY
B
10 μM Ginkgolic acid
C5-BODIPY
GA-BODIPY
1 5 10 1 5 10 (μM)
(kDa)
175
83
62
47.5
32.5
25
16.5
E1
RanGAP1-C2
(SUMO, E2)
WB : α-SUMO
WB : α-T7 (RanGAP1-C2)
WB : α-GST (E1)
WB : α-Ubc9 (E2)
C
E1
E2
GST
10 μM Ginkgolic acid
1 μM GA-BODIPY
10 μM GA-BODIPY
10 μM C5-BODIPY
10 μM GA-BODIPY
10 μM GA-BODIPY
(kDa)
175
83
62
47.5
32.5
25
16.5
E1
GST
E2
WB : α-GST (E1)
WB : α-Ubc9 (E2)
WB : α-GST (GST)
D
DMSO
Ginkgolic acid
Salicylic acid
Ac-GA
Me-GA
1 10 100 1 10 100 1 10 100 1 10 100 (μM)
GA-BODIPY
WB : α-GST (E1)
E
DTT (-)
DTT (+)
ATP (-)
DMSO
10 μM Ginkgolic acid
10 μM Anacardic acid
ATP (-)
DMSO
10 μM Ginkgolic acid
10 μM Anacardic acid
ATP (-)
DMSO
Ginkgolic acid
Anacardic acid
0.1 1 2.5 5 10 0.1 1 2.5 5 10 (μM)
(kDa)
160
105
75
50
40
30
25
20
E1-SUMO
Biotinated SUMO-1

Supplemental Data

Ginkgolic acid as an inhibitor of protein SUMOylation that blocks E1-SUMO intermediate formation

Isao Fukuda, Akihiro Ito, Go Hirai, Shinichi Nishimura, Hisashi Kawasaki, Hisato Saitoh, Ken-ichi Kimura, Mikiko Sodeoka, Minoru Yoshida

Supplementary Experimental Procedures

Antibodies

Goat polyclonal anti-SUMO-1 (N-19), goat polyclonal anti-UBC9 (N-15), mouse monoclonal anti-c-Myc (9E10), and goat polyclonal anti-p53 (FL393)-G antibodies were purchased from Santa Cruz Biotechnologies. Mouse monoclonal anti-T7, mouse monoclonal anti-p53 (Ab-6), and mouse monoclonal anti-GST (#3818-1) antibodies were from Novagen, Calbiochem, and Clontech, respectively. Mouse monoclonal anti-α-tubulin (B-5-1-2) and anti-FLAG (M2) antibodies were purchased from Sigma. Rabbit polyclonal anti-Histone H3, anti-acetyl H4 (Lys8), and rabbit polyclonal anti-acetyl-Lys antibodies were obtained from Cell Signaling.

Plasmids

Plasmids for His-tagged SUMO-1, His and T7-tagged RanGAP1-C2, GST-Aos1/Uba2, and His-tagged Ubc9 plasmids and biotinylated SUMO-1 were previously described (Uchimura et al., 2004). The plasmid containing cDNA for a p53 mutant having

arginine instead of lysine 386 (p53-K386R) or a E1 mutant having serine instead of cysteine 173 (E1 C173S) were generated by a site-direct mutagenesis using the wild-type human p53 plasmid (Ito et al., 2001) or the wild-type mouse E1 plasmid, respectively.

Bacterial protein expression and purification

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 (Uchimura et al., 2004) with minor modifications. Briefly, the pGEX and pET-based bacterial expression plasmids were introduced into *Escherichia coli* BL21 (DE3). The expression of recombinant proteins was induced with 0.2 mM isopropyl-β-D-galactopyranoside (IPTG) at 15°C for 24 h. Purification of GST- and $(His)_6$-fused proteins were carried out by either nickel (Ni^{2+})-affinity (QIAGEN) or glutathione-affinity (Amersham Biosciences). The protein concentration was determined by a Protein Assay Kit (Bio-Rad).

Cell culture, transfection and immunoblotting

293T and H1299 cells were maintained at 37°C in 5% CO_2 in Dulbecco's modified Eagles's medium (DMEM) supplemented with L-glutamine and 10% fetal bovine serum (FBS, GIBCO). 293T cells were seeded on 6-well plates and grown to approximately 60% confluence at the time of transfection. Two μg of DNA per well was used for transfection, using a Lipofectamine™ 2000 reagent (invitrogen). Cells were lysed in RIPA buffer (50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate, 5 mM EDTA, 0.1% Triton X-100) containing 50 mM

N-ethylmaleimide, a complete protease inhibitor cocktail tablet (Roche), and 1 mM PMSF. Proteins were separated by SDS-PAGE and transferred to a PVDF membrane (Millipore) by electroblotting. After the membranes had been incubated with primary and secondary antibodies, the immune complexes were detected with an Immobilon Western kit (Millipore), and the luminescence was analyzed with a LAS-3000 image analyzer (Fujifilm).

Immunoprecipitation

p53-null H1299 cells were co-transfected with 1 μg of the expression vectors for Flag-SUMO-1 and p53 wt or the K386R mutant, using Lipofectamine^{TM} 2000 (Invitrogen). For the detection of the SUMOylated forms of p53, cells were lysed in RIPA buffer containing 50 mM *N*-ethylmaleimide by rotating for 30 min at 4°C. The lysates were incubated with an anti-p53 (FL393)-G antibody in RIPA buffer for 1 h at 4°C and the immune complexes were captured with protein A-agarose beads for 1 h at 4°C. Bead-bound proteins were washed three times with RIPA buffer, and immunoprecipitates were eluted and analyzed by immunoblotting.

***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 mixed with reducing/loading buffer, and then 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).

GA-BODIPY-binding assay

GA-BODIPY or C5-BODIPY (1-100 μM) was added to the *in vitro* SUMOylation reaction mixture or to buffer (50 mM Tris (pH 7.4), 6 mM $MgCl_2$, 2 mM ATP) containing 0.3 μg of GST-Aos1/Uba2 (E1), 0.1 μg of His-tagged Ubc9 (E2), or 0.2 μg of GST. The mixtures were incubated with SDS loading buffer in the absence of 2-mercaptoethanol and loaded without boiling onto an 11% SDS-polyacrylamide gel. The binding of GA-BODIPY to E1 was detected using a fluorescence detector with excitation at 488 nm.

***In vitro* HAT assay**

Twenty μg/ml of calf thymus histone was incubated with 2 μg/ml GST-PCAF in the presence of 0.1 mM acetyl CoA in the 50 μl HAT buffer (50 mM Tris-HCl pH 8.0, 0.1 mM EDTA, 1 mM DTT and 10% glycerol) for 1 h at 30°C. Samples were mixed with reducing/loading buffer, and then separated by on 15% SDS-PAGE followed by immunoblotting using an anti-acetyl lysine antibody.

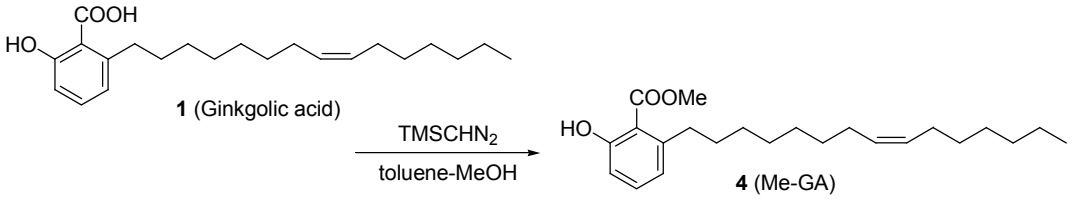
WST-1 assay

HeLa cells were seeded in triplicate into 96-well plates at a density of 5,000 cells/well, and were then treated with various concentrations of ginkgolic acid, Ac-GA, or Me-GA (25-100 μM) for 48 h. The viable cells were stained with WST-1 (Dojindo). Plates were analyzed in a microtiter plate reader at 450 nm with a reference wavelength of 650 nm.

Synthesis of Methyl (*Z*)-2-hydroxy-6-(pentadec-8-enyl)benzoate (Me-GA 4) and (*Z*)-2-Acetoxy-6-(pentadec-8-enyl)benzoic acid (Ac-GA 5).

General; NMR spectra were recorded on a JEOL JNM-AL400 spectrometer, operating at 400 MHz for ^{1}H-NMR. Chemical shifts were reported in the scale relative to $CDCl_3$ as an internal reference. MALDI-TOFMS was taken on BrukerDaltonics Microflex with matrix dimer and angiotensin I as internal standards.

Methyl (*Z*)-2-hydroxy-6-(pentadec-8-enyl)benzoate (Me-GA **4**)

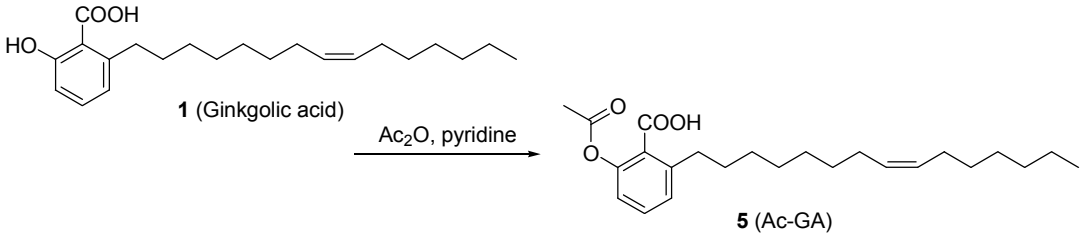


To a solution of the gingkolic acid (1.1 mg, 3.17 μmol) in toluene (350 μl) and MeOH (100 μl) was added trimethylsilyldiazomethane (2.0 M solution in diethyl ether, 30 μl) at room temperature. After stirring for 30 min at room temperature, the reaction mixture was concentrated in vacuo. Further purification was carried out by HPLC (performed on Waters 600 HPLC Pump with Waters 600 LCD Controller measured at 310 nm; column,

Wakopak 4.6 mm x 250 mm; mobile phase, MeOH/H_2O = 9/1 (1% acetic acid); RT = 38 min) to give **4** (0.7 mg, 61%) as a white amorphous.

^{1}H-NMR (400 MHz, $CDCl_3$) δ 0.89 (t, *J* = 7.1 Hz, 3H), 1.24-1.39 (m, 18H), 2.02 (m, 4H), 2.88 (br t, *J* = 7.8 Hz, 2H), 3.96 (s, 3H), 5.35 (m, 2H), 6.72 (d, *J* = 8.0 Hz, 1H), 6.84 (d, *J* = 8.0 Hz, 1H), 7.29 (t, *J* = 8.0 Hz, 1H), 11.1 (s,1H); MALDI-TOF/MS (positive ion, α-cyano-4-hydroxy-cinnamic acid) calcd. for $C_{23}H_{36}O_3Na$ ($M+Na^+$) 383.256; found 383.264.

(*Z*)-2-Acetoxy-6-(pentadec-8-enyl)benzoic acid (Ac-GA **5**)



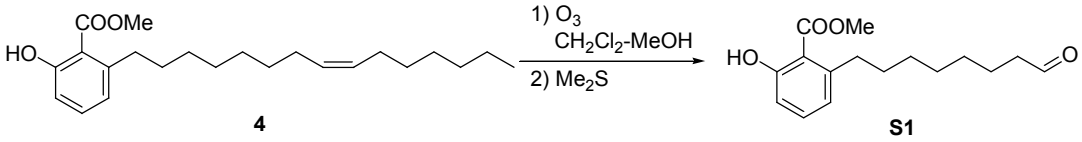
To a solution of the gingkolic acid (1.0 mg, 2.89 μmol) in pyridine (100 μl) was added acetic anhydride (50 μl) at room temperature. After stirring for 1 day at room temperature, the reaction mixture was diluted with $CHCl_3$, and a 1N aqueous solution of HCl was added. The organic layer was separated, and the aqueous layer was extracted with $CHCl_3$. The combined organic layers were dried over Na_2SO_4 and concentrated in vacuo. Further purification was carried out by PTLC (hexane/ethyl ecetate = 1/1) to give **5** (0.6 mg, 55%) as a white amorphous.

^{1}H-NMR (400 MHz, $CDCl_3$) δ 0.89 (t, *J* = 6.4 Hz, 3H), 1.23-1.39 (m, 18H), 2.02 (m, 4H), 2.29 (s, 3H), 2.75 (br t, *J* = 7.8 Hz, 2H), 5.35 (m, 2H), 6.97 (d, *J* = 7.8 Hz, 1H), 7.14 (d, *J* = 7.8 Hz, 1H), 7.37 (t, *J* = 7.8 Hz, 1H); MALDI-TOF/MS (positive ion,

α-cyano-4-hydroxy-cinnamic acid) calcd. for $C_{24}H_{36}O_4Na$ ($M+Na^+$) 411.251; found. 411.281.

Synthesis of **GA-BODIPY 6**

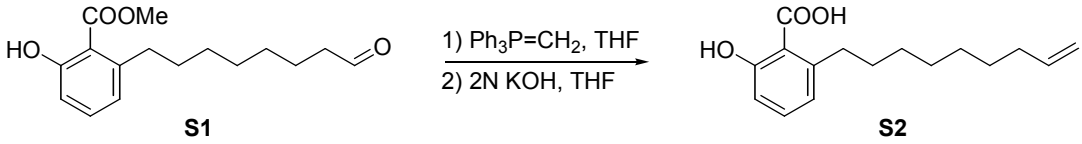
Synthesis of aldehyde **S1**.



4 **S1**

Ozone was bubbled through a solution of **4** (15.6 mg, 38.7 μmol) in CH_2Cl_2-MeOH (500 μl-500μl) at -78 °C for 1 min. After argon was bubbled through a mixture at -78 °C for 10 min, Me_2S (28.4 ml, 0.387 mmol) was added to the mixture. The mixture was warmed to room temperature and stirred for 1 h, and H_2O (10 ml) was added. The mixture was extracted with Et_2O twice. The combined organic layers were dried over Na_2SO_4 and concentrated in vacuo to give an aldehyde **S1** (31.2 mg, mixture with phthalate).

Synthesis of alkene **S2**.



S1 **S2**

To a mixture of methyltriphenylphosphonium bromide (41.5 mg, 116.1 μmol) in THF (0.5 ml) was added potassium *t*-butoxide (12.8 mg, 114.2 μmol) at 0 °C. After stirring for 30 min, the **S1** (crude 31.2 mg) in THF (0.5 ml) was added at 0 °C. The mixture was warmed to room temperature and stirred for 1 h, and H_2O (10 ml) was added. The mixture was extracted with Et_2O twice. The combined organic layers were dried over

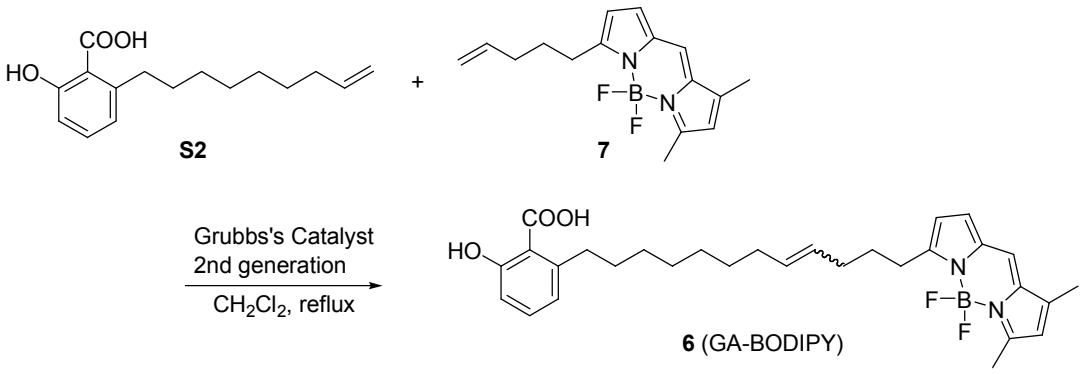
Na_2SO_4 and concentrated in vacuo. Further purification was carried out by PTLC (hexane/ethyl ecetate = 3/1) to give alkene-methyl ester (18.7 mg, mixture with phthalate) and aldehyde **S1** (2.8 mg, 26%).

S1: ^{1}H-NMR (400 MHz, $CDCl_3$) δ 1.20-1.64 (m, 10H), 2.38 (dt, *J* = 7.3 Hz, 1.4 Hz, 2H), 2.82 (br t, *J* = 7.6 Hz, 2H), 3.88 (s, 3H), 6.66 (d, *J* = 7.8 Hz, 1H), 6.79 (d, *J* = 7.8 Hz, 1H), 7.24 (t, *J* = 7.8 Hz, 1H), 9.71 (br s, 1H), 11.05 (s, 1H).

To a mixture of alkene-methyl ester (18.7 mg) in THF (500 μl) was added a 2M aqueous solution of KOH (100 μl) at room temperature. After stirring for 2 days at refluxing temperature, the reaction mixture was diluted with $CHCl_3$ and a 1 N aqueous solution of HCl was added. The aqueous layer was separated and extracted with $CHCl_3$ twice. The combined organic layers were dried over Na_2SO_4 and concentrated in vacuo. Further purification was carried out by silica gel column chromatography ($CHCl_3$ / MeOH = 30 / 1) to give **S2** (3.0 mg, 44% from **4**) as a white amorphous.

S2: ^{1}H-NMR (400 MHz, $CDCl_3$) δ 1.20-1.44 (m, 8H), 1.60 (m, 2H), 2.04 (dt, *J* = 7.1 Hz, 7.1 Hz, 2H), 2.97 (br t, *J* = 7.6 Hz, 2H), 4.93 (d, *J* = 9.8 Hz, 1H), 4.99 (d, *J* = 17.3 Hz, 1H), 5.81 (ddt, *J* = 17.3 Hz, 9.8 Hz, 7.1 Hz, 1H), 6.77 (d, *J* = 7.8 Hz, 1H), 6.87 (d, *J* = 7.8 Hz, 1H), 7.34 (t, *J* = 7.8 Hz, 1H), 10-12 (br).

Synthesis of GA-BODIPY **7**.



To a mixture of **S2** (3.0 mg, 10.9 μmol) and **7** (4.8 mg, 18.5 μmol) in CH_2Cl_2 (1 ml) was added Grubbs catalyst 2nd generation (Aldrich, 0.9 mg, 1.09 μmol) at room temperature. After stirring for 12 h at refluxing temperature, triethylamine (1 ml) was added to the reaction mixture. The resulting mixture was concentrated in vacuo. Further purification was carried out by silica gel column chromatography ($CHCl_3$ / MeOH = 30 / 1) and PTLC ($CHCl_3$ / MeOH = 9 / 1) to give **6** (3.2 mg, 56%) as a dark orange amorphous.

^{1}H-NMR (400 MHz, $CDCl_3$) δ 1.23-1.42 (m, 8H), 1.44-1.65 (m, 2H), 1.79 (quin, J = 8.1 Hz, 2H), 1.95-2.16 (m, 4H), 2.24 (s, 3H), 2.56 (s, 3H), 2.95 (m, 4H), 5.38-5.50 (m, 2H), 6.09 (s, 1H), 6.28 (d, J = 3.4 Hz, 1H), 6.74 (d, J = 8.0 Hz, 1H), 6.84 (d, J = 8.0 Hz, 1H), 6.90 (d, J = 3.4 Hz, 1H), 7.06 (s, 1H), 7.32 (t, J = 8.0 Hz, 1H); MALDI-TOF/MS (positive ion, α-cyano-4-hydroxy-cinnamic acid) calcd. for $C_{30}H_{37}BF_2N_2O_3Na$ ($M+Na^+$) 545.276; found. 545.415.

Supplemental References

Ito, A., Lai, C. H., Zhao, X., Saito, S., Hamilton, M. H., Appella, E., and Yao, T. P. (2001). p300/CBP-mediated p53 acetylation is commonly induced by p53-activating agents and inhibited by MDM2. Embo J. *20*, 1331-1340.

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.

Figure Legends

Figure S1. Effects of ginkgolic acid and anacardic acid on time-dependent *in vivo* SUMOylation and ubuiquination

(**A**) Inhibition of *in vivo* protein SUMOylation by ginkgolic acid and anacardic acid. 293T cells that had been transfected with Flag-tagged SUMO were treated with 100 μM of ginkgolic acid or anacardic acid for indicated times (1-8 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. (**B**) Effects of ginkgolic acid and anacardic acid on *in vivo* ubiquitination. 293T cells that had been transfected with Myc-tagged ubiquitin were treated with 100 μM of ginkgolic acid or anacardic acid for indicated times (1-8 h) in the presence of 10 μM of the proteasome inhibitor MG132 (in order to increase the level of ubiquitination). 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-Myc antibody.

Figure S2. Inhibition of protein SUMOylation by GA-BODIPY

Various concentrations of C5-BODIPY and GA-BODIPY (1-10 μM) were added to the SUMOylation reaction mixture containing His-tagged SUMO-1, His- and T7-tagged RanGAP1-C2, GST-Aos1/Uba2 (E1), His-tagged-Ubc9 (E2) in the presence of ATP, and then SUMOylated RanGAP1-C2 was detected by immunoblotting using an anti-T7 or anti-SUMO-1 antibody.

Figure S3. Specific binding of GA-BODIPY to E1 under denaturing conditions.

GA-BODIPY or C5-BODIPY at various concentrations (1-10 μM) was added to the SUMOylation reaction mixture containing His-tagged SUMO-1, His and T7-tagged RanGAP1-C2, GST-Aos1/Uba2, His-tagged Ubc9. The proteins were separated by SDS-PAGE under either nondenaturing conditions (without 2-mercaptoethanol and boiling) or denaturing conditions (with 2-mercaptoethanol and boiling). The bound protein was detected using a fluorescence detector with excitation at 488 nm following SDS-PAGE.

Figure S4. Inhibitory effects of ginkgolic acid and anacardic acid on HAT activity *in vitro* and *in vivo*

(**A**) Inhibition of PCAF-mediated histone acetylation by ginkgolic acid and anacardic acid *in vitro*. Various concentrations of ginkgolic acid and anacardic acid (1-25 μM) were added to the reaction mixture containing calf thymus histone, GST-PCAF, and acetyl CoA, and then acetylated histones were detected by immunoblotting using an anti-acetyl lysine antibody. (**B**) Effects of ginkgolic acid and anacardic acid on *in vivo* histone acetylation. 293T cells were treated with various concentrations of ginkgolic acid and anacardic acid (10-100 μM) for 8 h. Cells were lysed in RIPA buffer and the lysates were separated by 15% SDS-PAGE followed by immunoblotting using an anti-acetyl H4 (Lys8) antibody.

Figure S5. Effects of ginkgolic acid, Ac-GA, and Me-GA on the viability of HeLa cells

HeLa cells were treated with various concentrations (25-100 μM) of the compounds for

48 h. Viability of cells was determined by the WST-1 assay as described in Supplementary Methods using a microtiter plate reader. Data are the means ± SD from three independent assays.

Figure S6. The binding of GA-BODIPY to the active-site E1 mutant

(**A**) *In vitro* SUMOylation assay using the E1 C173S. SUMOylation reaction mixture containing His-tagged SUMO-1, His and T7-tagged RanGAP1-C2, His-tagged-Ubc9 (E2), and either GST-Aos1/Uba2 wild-type (E1 wt) or C173S mutant (E1 C173S) in the presence of ATP, and then SUMOylated RanGAP1-C2 was detected by immunoblotting using an anti-SUMO-1 antibody. (**B**) The binding of GA-BODIPY to the E1 C173S mutant. Either E1 wt or the C173S mutant was incubated with GA-BODIPY, C5-BODIPY, or ginkgolic acid at 10 μM in the presence of ATP. The E1 proteins were separated by SDS-PAGE under nondenaturing conditions (without 2-mercaptoethanol and boiling). The BODIPY-bound proteins were detected using a fluorescence detector.

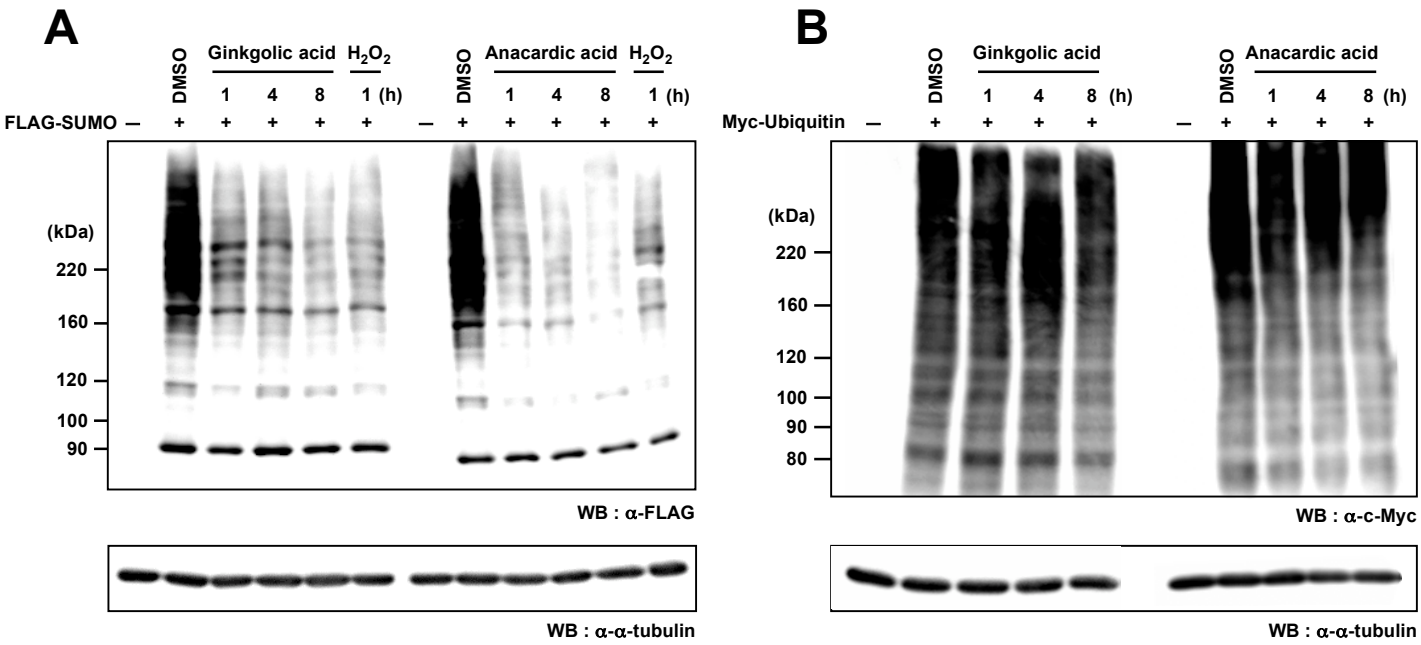

A
DMSO
Ginkgolic acid
H_2O_2
1 4 8 1 (h)
DMSO
Anacardic acid
H_2O_2
1 4 8 1 (h)
FLAG-SUMO — + + + + + — + + + + +
(kDa)
220
160
120
100
90
WB : α-FLAG
WB : α-α-tubulin
B
DMSO
Ginkgolic acid
1 4 8 (h)
DMSO
Anacardic acid
1 4 8 (h)
Myc-Ubiquitin — + + + + — + + + +
(kDa)
220
160
120
100
90
80
WB : α-c-Myc
WB : α-α-tubulin

Figure S1

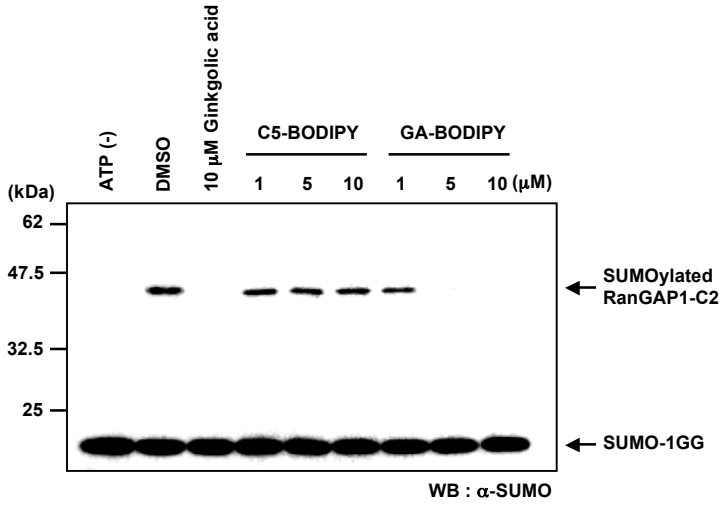

ATP (-)
DMSO
10 μM Ginkgolic acid
C5-BODIPY
GA-BODIPY
1
5
10
1
5
10 (μM)
(kDa)
62
47.5
32.5
25
SUMOylated
RanGAP1-C2
SUMO-1GG
WB : α-SUMO

Figure S2

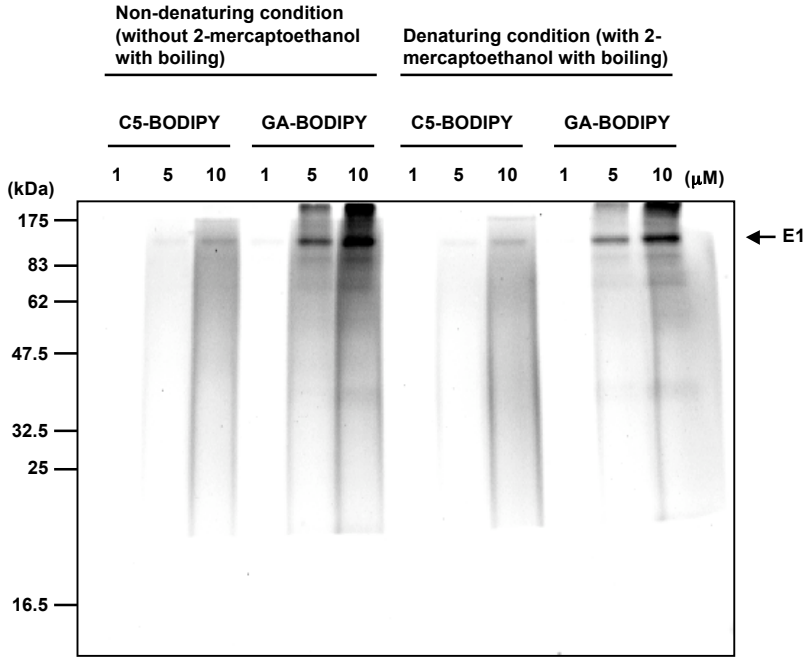
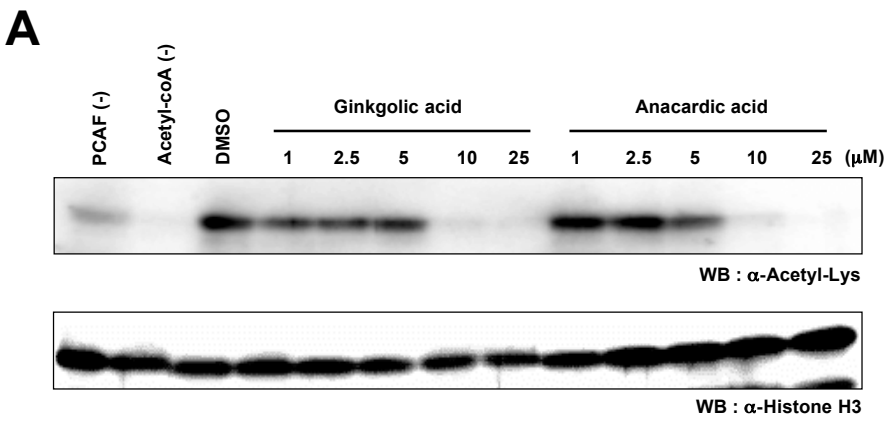

Non-denaturing condition (without 2-mercaptoethanol with boiling)
Denaturing condition (with 2-mercaptoethanol with boiling)
C5-BODIPY
GA-BODIPY
C5-BODIPY
GA-BODIPY
1 5 10 1 5 10 1 5 10 1 5 10 (μM)
(kDa)
175
83
62
47.5
32.5
25
16.5
E1

Figure S3


A
PCAF (-)
Acetyl-coA (-)
DMSO
Ginkgolic acid
Anacardic acid
1 2.5 5 10 25 1 2.5 5 10 25 (μM)
WB : α-Acetyl-Lys
WB : α-Histone H3

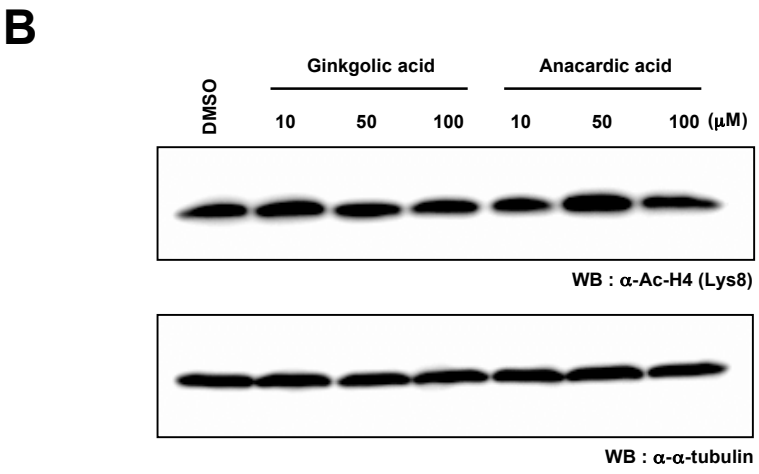

B
DMSO
Ginkgolic acid
Anacardic acid
10 50 100 10 50 100 (μM)
WB : α-Ac-H4 (Lys8)
WB : α-α-tubulin

Figure S4

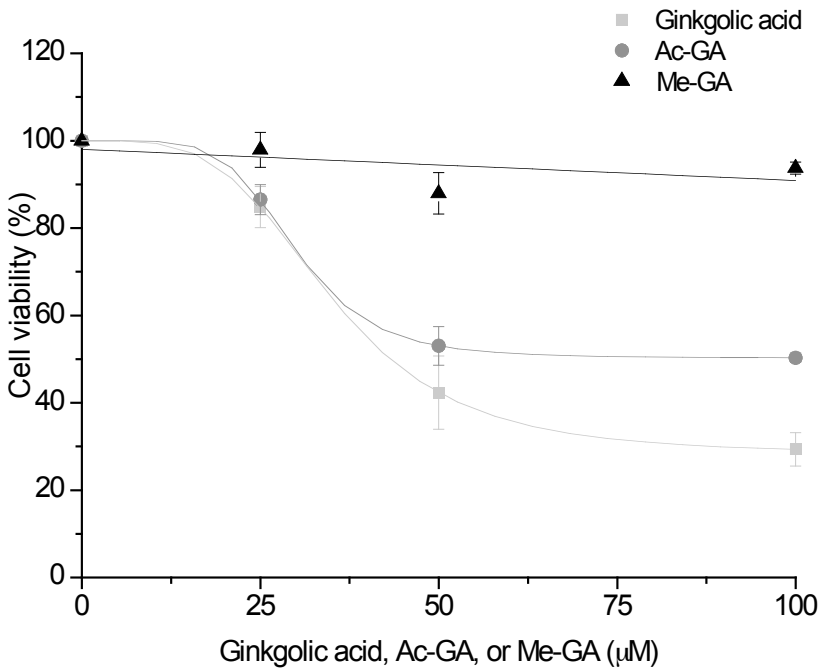
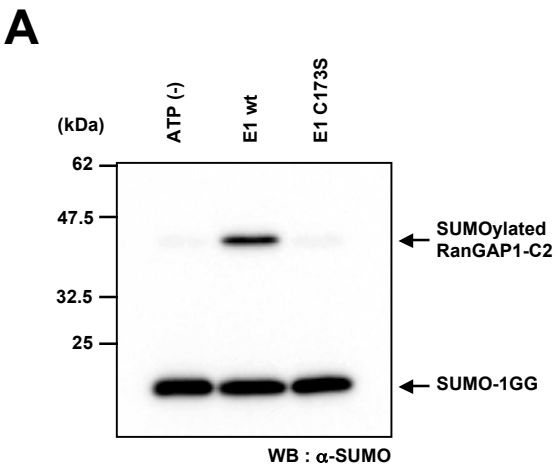

Ginkgolic acid
Ac-GA
Me-GA
Cell viability (%)
120
100
80
60
40
20
0
0
25
50
75
100
Ginkgolic acid, Ac-GA, or Me-GA (μM)

Figure S5


A
ATP (-)
E1 wt
E1 C173S
(kDa)
62
47.5
32.5
25
SUMOylated
RanGAP1-C2
SUMO-1GG
WB : α-SUMO

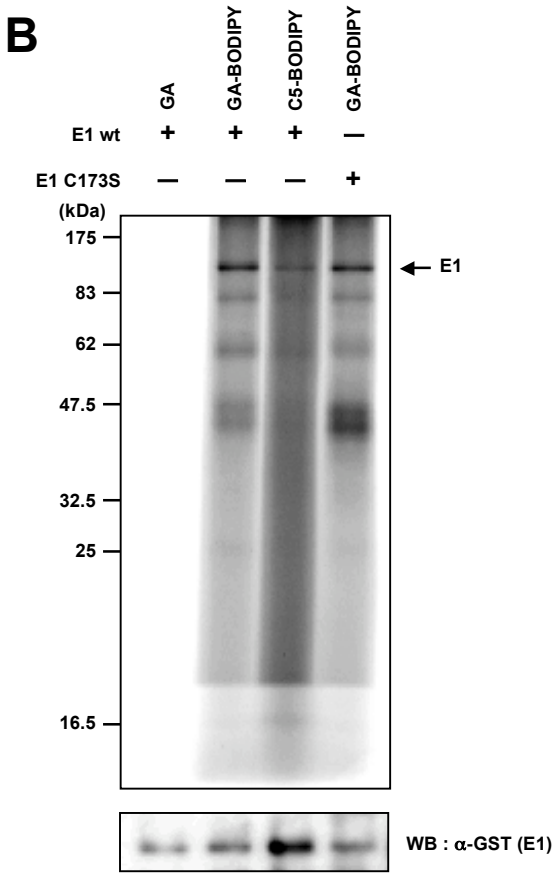

B
GA
GA-BODIPY
C5-BODIPY
GA-BODIPY
E1 wt
E1 C173S
(kDa)
175
83
62
47.5
32.5
25
16.5
E1
WB : α-GST (E1)

Figure S6