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Letters

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Spectomycin B1 as a Novel SUMOylation Inhibitor That Directly Binds to SUMO E2

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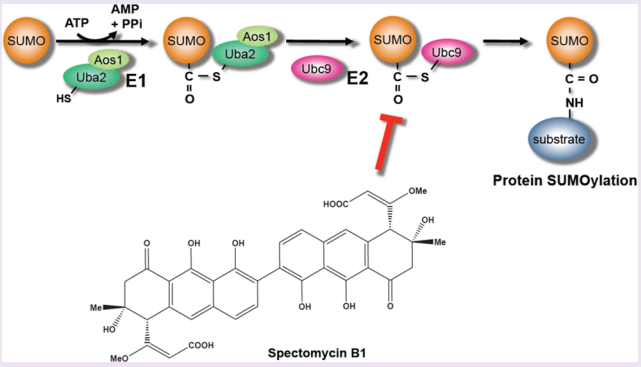
Supporting Information

ABSTRACT: Conjugation of small ubiquitin-like modifier (SUMO) to protein (SUMOylation) regulates multiple biological systems by changing the functions and fates of a large number of proteins. Consequently, abnormalities in SUMOylation have been linked to multiple diseases, including breast cancer. Using an *in situ* cell-based screening system, we have identified spectomycin B1 and related natural products as novel SUMOylation inhibitors. Unlike known SUMOylation inhibitors such as ginkgolic acid, spectomycin B1 directly binds to E2 (Ubc9) and selectively blocks the formation of the E2-SUMO intermediate; that is, Ubc9 is the direct target of spectomycin B1. Importantly, either spectomycin B1 treatment or Ubc9 knockdown inhibited estrogen-dependent proliferation of MCF7 human breast-cancer cells. Our findings suggest that Ubc9 inhibitors such as spectomycin B1 have potential as therapeutic agents against hormone-dependent breast cancers.



SUMOylation, a major posttranslational modification, alters the functions and fates of diverse proteins by changing their subcellular localizations, protein−protein interactions, stabilities, or catalytic activities, thereby regulating multiple biological systems including transcription, intracellular transport, DNA repair, replication, and cell signaling.[1] SUMOylation is catalyzed by a multistep enzymatic cascade similar to that of ubiquitination.[1] In the initial step, the SUMO precursor protein is cleaved by the carboxyl-terminal hydrolase activity of SUMO-specific isopeptidases (SENPs), generating a free carboxyl-terminal diglycine motif that is necessary for SUMO conjugation to target proteins. SUMO is then activated by ATP-dependent thioester-bond formation between its C-terminal glycine and the catalytic cysteine residue in SUMO-activating enzyme E1 (a heterodimer consisting of Aos1 and Uba2, also named SAE1 and SAE2). Next, SUMO is transferred from E1 to the catalytic cysteine residue in a SUMO-conjugating enzyme called E2 (also known as Ubc9) via a thioester linkage. Although multiple E2 enzymes have been identified in the ubiquitination pathway, Ubc9 is the sole E2 required for SUMOylation. In the last step, SUMO is transferred from E2 to the ε-amino group of a lysine residue on a substrate protein. SUMO E3 ligases are able to enhance the translocation of SUMO from Ubc9 to target proteins and also contribute to substrate specificity. Like ubiquitination, SUMOylation is reversible; SUMO can be cleaved from target proteins by SENPs.[1]

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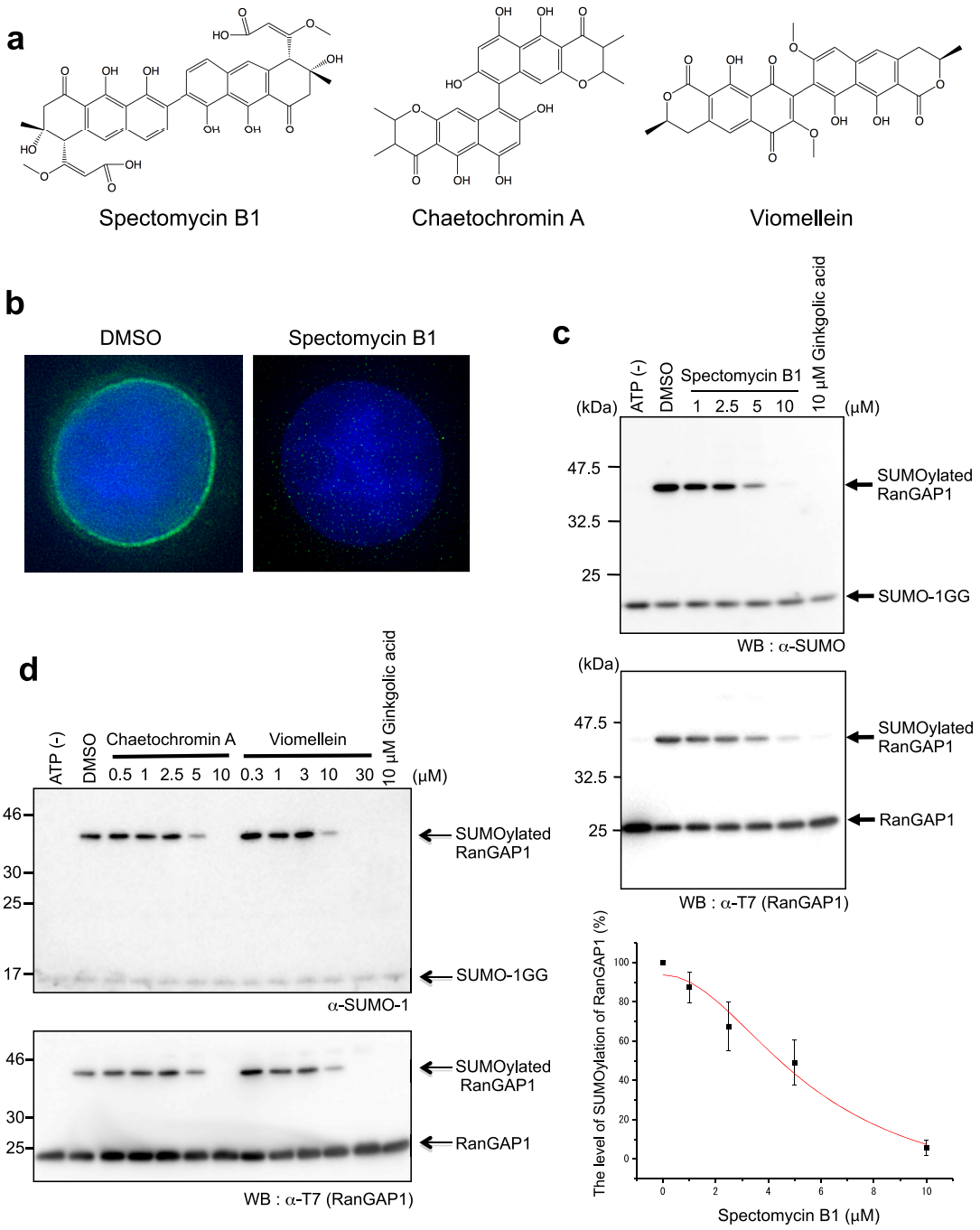

Figure 1. Spectomycin B1 inhibits SUMOylation *in vitro*. (a) Structures of spectomycin B1, chaetochromin A, and viomellein. (b) Inhibition of *in situ* SUMOylation by spectomycin B1. Digitonin-permeabilized HeLa cells were incubated with GFP and His-tagged SUMO-1, GST-fused Aos1/Uba2 (E1), His-tagged Ubc9 (E2), and ATP in the presence or absence of 14 μM spectomycin B1, and then GFP signal was observed under a fluorescent microscope. (c) Inhibition of *in vitro* SUMOylation by spectomycin B1. Various concentrations of the spectomycin B1 (1−10 μM) were incubated with the SUMOylation reaction mixture containing His-tagged SUMO-1, His- and T7-tagged RanGAP1-C2, GST-fused Aos1/Uba2 (E1), and His-tagged Ubc9 (E2) in the presence of ATP. SUMOylated RanGAP1 was detected by immunoblotting using an anti-T7 or an anti-SUMO-1 antibody. Ginkgolic acid was used as a positive control.[14] The levels of SUMOylated RanGAP1 were determined by measuring the intensity of SUMOylated RanGAP1 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 based on 50% inhibition compared with a control sample containing none of these compounds. (d) Inhibition of *in vitro* SUMOylation by chaetochromin A and viomellein. SUMOylated RanGAP1 was detected as described in part c.

Abnormal SUMO modifications have been implicated in a variety of disorders, including cancers. Several tumor suppressors and oncogenic proteins are targets for SUMOylation.[2,3] For instance, multiple DNA repair proteins undergo Ubc9-mediated SUMO conjugations that affect the DNA-damage response (DDR), which is important for genome integrity; aberrations in the DDR are linked to development of malignancies.[4] Remarkably, increased expression of enzymes responsible for SUMO-conjugation pathways, including Ubc9, have been observed in multiple types of cancers, e.g., ovarian carcinoma,[5] lung adenocarcinoma,[6] metastatic prostate cancer,[7] progressive melanoma,[8] and breast tumors.[9] In addition, a

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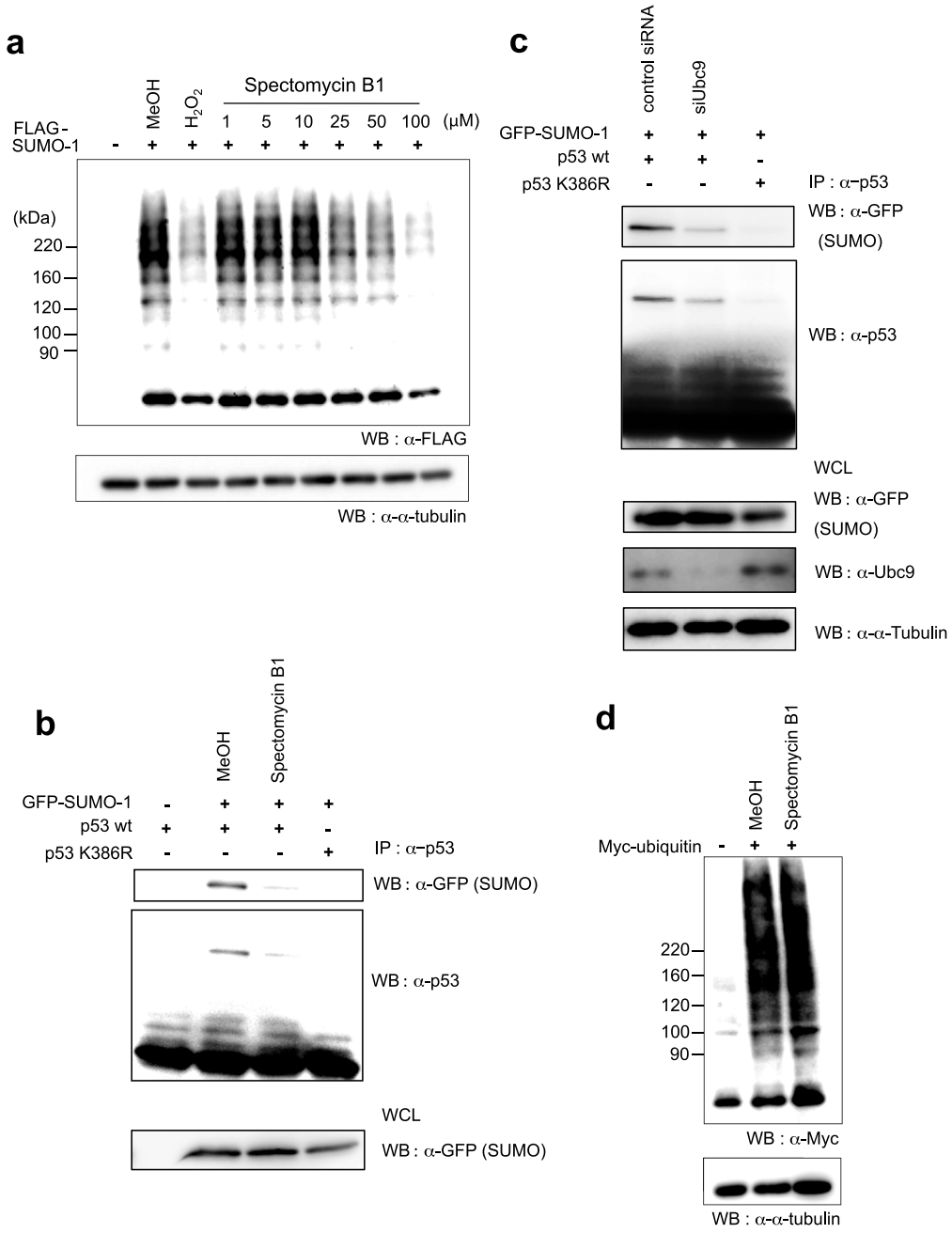

Figure 2. Spectomycin B1 inhibits SUMOylation *in vivo*. (a) Inhibition of *in vivo* protein SUMOylation by spectomycin B1. 293T cells were transfected with an expression vector containing FLAG-tagged SUMO-1, and then treated with various concentrations of spectomycin B1 (1−100 μM) for 12 h or with 1 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 with anti-FLAG or anti-α-tubulin antibody. (b) Inhibition of *in vivo* p53 SUMOylation by spectomycin B1. H1299 cells ($p53^{-/-}$) transfected with the indicated combinations of GFP-tagged SUMO, wild-type p53, and SUMOylation-deficient K386R mutant p53 were treated with 100 μM of spectomycin B1 for 24 h. Cell extracts were immunoprecipitated with an anti-p53 (FL393-G) antibody followed by immunoblotting with anti-GFP or anti-p53 (AB-6) antibody. (c) Effect of Ubc9 knockdown on *in vivo* p53 SUMOylation. H1299 cells were transfected with control or siRNA oligos targeting *Ubc9* mRNA, and SUMOylated p53 expressed in the cells was detected as described in part b. (d) Effects of spectomycin B1 on *in vivo* ubiquitination. 293T cells were transfected with an expression vector containing a myc-tagged ubiquitin cDNA, and then treated with 100 μM of spectomycin B1 for 12 h in the presence of 10 μM of the proteasome inhibitor MG132. Cells were lysed in RIPA buffer containing 50 mM *N*-ethylmaleimide, and the lysates were separated by 6% SDS-PAGE followed by immunoblotting using anti-c-myc or anti-α-tubulin antibody.

recent study indicated that polymorphisms in the *Ubc9* gene were associated with the tumor grade of breast carcinoma.[10] Furthermore, antagonizing Ubc9 function with a dominant-negative mutant suppressed the estrogen receptor (ER) α-dependent signal pathway in MCF7 breast-cancer cells and reduced tumor growth in a xenograft model using MCF7 cells.[11] Furthermore, a defective mutation in *Ubc9* enhances cellular sensitivity to genotoxic agents.[12] These observations suggest that enzymes responsible for SUMO conjugation pathways, including Ubc9, are potential targets for cancer therapy, and that inhibition of Ubc9 by small-molecule

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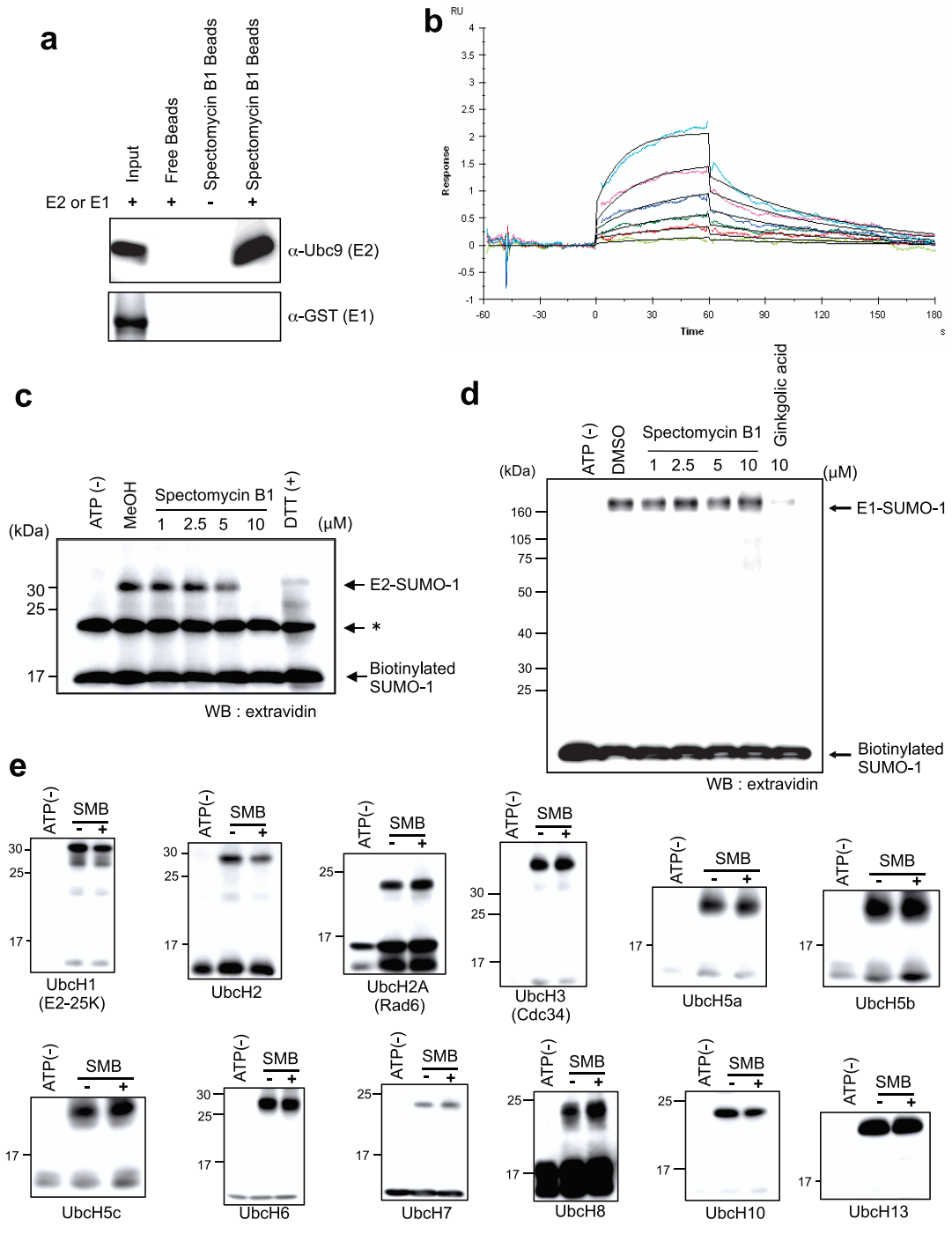

Figure 3. Ubc9 is the target protein of spectomycin B1. (a) Spectomycin B1-conjugated beads selectively pulled down Ubc9 protein. Spectomycin B1-conjugated beads or control beads were incubated with His-tagged Ubc9 protein or GST-tagged Aos1/Uba2 protein for 1 h. Bound proteins were separated by SDS-PAGE followed by immunoblotting using anti-Ubc9 or anti-GST antibody. (b) Sensorgram of spectomycin B1 binding to immobilized Ubc9. Spectomycin B1 concentrations used, in descending order of response units (RU), were 20, 10, 5, 2.5, 1.25, 0.625 μM. (c) Inhibition of the thioester bond formation between Ubc9 and biotinylated SUMO-1 by spectomycin B1. Various concentrations of spectomycin B1 (1−10 μM) were added to a reaction mixture containing biotinylated SUMO1 proteins, GST-Aos1/Uba2 proteins and His-tagged Ubc9 proteins in the presence or absence of 2 mM ATP. The mixtures were separated by SDS-PAGE, followed by analysis using avidin-conjugated horseradish peroxidase. Addition of 1 mM DTT abolished formation of the Ubc9-biotinylated SUMO-1 intermediate. (d) Effects of spectomycin B1 on thioester-bond formation between E1 and biotinylated SUMO-1. Various concentrations of spectomycin B1 (1−10 μM) were added to a reaction mixture containing biotinylated SUMO-1 proteins and GST-Aos1/Uba2 proteins in the presence or absence of 2 mM ATP. The mixtures were separated by SDS-PAGE followed by analysis using avidin-conjugated horseradish peroxidase. Ginkgolic acid was used as a positive control.[14] (e) Effects of spectomycin B1 on thioester-bond formation between a series of ubiquitin E2s and biotinylated ubiquitin. Spectomycin B1 (10 μM) was added to a reaction mixture containing biotinylated ubiquitin and indicated ubiquitin E2 in the presence or absence of ATP. Ubiquitin-E2 thioester bonds were detected by immunoblot with avidin-conjugated horseradish peroxidase.

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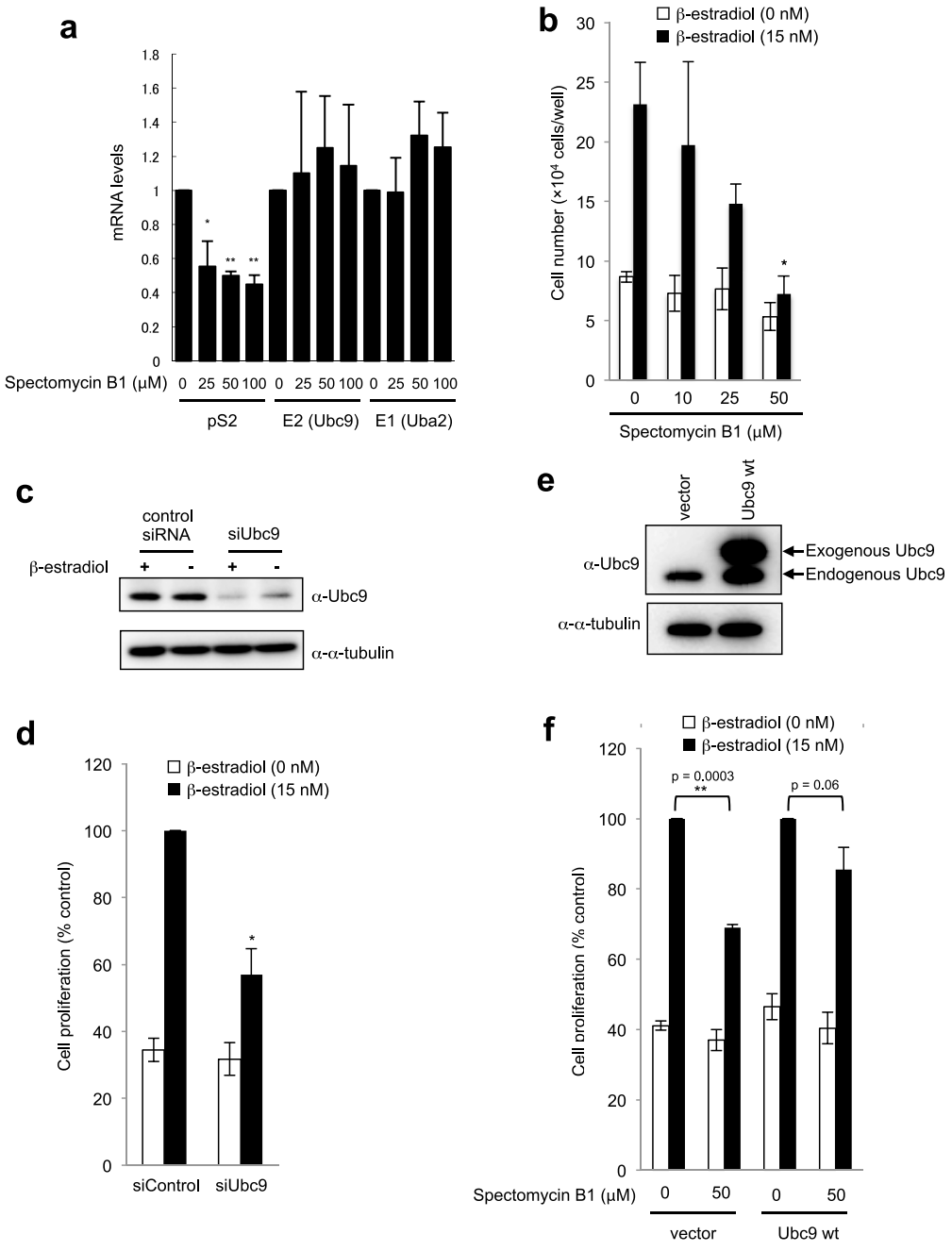

Figure 4. Spectomycin B1 inhibits ERα-regulated gene expression and growth of breast-cancer cells. (a) Downregulation of *pS2* mRNA expression by spectomycin B1. MCF7 cells were treated with various concentrations of spectomycin B1 for 24 h and mRNA levels of the indicated genes were measured by quantitative real-time RT-PCR. The mRNA levels of all genes were normalized against β-actin as an internal control. *P < 0.05, **P < 0.01. (b) Inhibition of β-estradiol-induced proliferation of MCF7 cells by spectomycin B1. MCF7 cells were treated with various concentrations of spectomycin B1 and 15 nM of β-estradiol for 96 h and living cells were counted. *P < 0.05. (c and d) Effects of Ubc9 knockdown on β-estradiol-induced cell proliferation. MCF7 cells were transfected with control or siRNA oligos targeted against *Ubc9* mRNA, followed by treatment with β-estradiol for 96 h. Cell lysates after treatment with siRNA oligos for 72 h were separated by 14% SDS-PAGE, followed by immunoblotting using anti-Ubc9 or anti-α-tubulin antibody (c). Cell proliferation was measured by the WST-1 assay (d). Data are representative of three independent determinations. All error bars represent standard deviation of the mean. *P < 0.05. (e and f) Overexpression of Ubc9 counteracts the cell-growth inhibition by spectomycin B1. MCF7 cells stably expressing empty vector or myc-tagged Ubc9 using a retrovirus system were treated with 50 μM spectomycin B1 and 15 nM β-estradiol for 96 h. Protein levels of Ubc9 and α-tubulin and proliferation of MCF7 cells were measured as described in parts c and d, respectively. Data are representative of three independent determinations. All error bars represent standard deviation of the mean. **P < 0.01. When cells were transfected with an empty vector, β-estradiol-induced proliferation differed statistically significantly between samples treated with DMSO and spectomycin B1, but there was no such difference between DMSO- and spectomycin B1-treated samples when cells were expressing Ubc9.

compounds represents a potential strategy for tackling SUMO-dependent cancers.[13]

Notwithstanding the importance of SUMOylation in regulating diverse biological systems and diseases, only a few

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small-molecule inhibitors of SUMOylation have been reported to date. Previously, we identified ginkgolic acid as the first known small-molecule SUMOylation inhibitor.[14] To identify additional small-molecule inhibitors of protein SUMOylation, we screened a chemical library that includes natural products using an *in situ* SUMOylation assay (Supporting Information (SI) Figure S1) and reported another inhibitor, kerriamycin B.[15] These natural products act as SUMO E1 inhibitors, as they inhibit the E1-SUMO intermediate formation. Using the same system, we found that spectomycin B1 (Figure 1a), a compound produced by *Streptomyces spectabilis* that was originally identified as an antibiotic against gram-positive bacteria,[16] inhibits protein SUMOylation (Figure 1b). An *in vitro* SUMOylation assay using RanGAP1-C2 as substrate demonstrated that spectomycin B1 inhibits *in vitro* SUMOylation in a dose-dependent manner and that its IC_{50} value is 4.4 μM (Figure 1c). Next, we tested whether chaetochromin A and viomellein (Figure 1a), natural products structurally related with spectomycin B1, also inhibit SUMOylation. Indeed, chaetochromin A and viomellein inhibited *in vitro* SUMOylation of RanGAP1-C2 with IC_{50} values of 3.7 μM and 10.2 μM, respectively (Figure 1d and SI Figure S2). Due to the limited supply of these natural products, we further characterized this class of SUMOylation inhibitors using only spectomycin B1. To test the effect on *in vivo* SUMOylation, we analyzed the levels of SUMOylated proteins in 293T cells expressing FLAG-tagged SUMO-1. The amount of high-molecular weight SUMO conjugates was reduced by spectomycin B1 in a dose-dependent manner (Figure 2a). Hydrogen peroxide also decreased the level of SUMO conjugates, as previously reported.[17] We next examined whether spectomycin B1 affects *in vivo* SUMOylation of p53. Spectomycin B1 as well as Ubc9 knockdown by siRNA oligos markedly reduced the level of SUMOylation on lysine residue 386, the major site of p53 SUMOylation (Figure 2b and c).[18,19] Importantly, spectomycin B1 had no effect on the cellular level of ubiquitinated proteins (Figure 2d). These results indicate that spectomycin B1 inhibits protein SUMOylation without affecting protein ubiquitination in cells.

Because spectomycin B1 inhibits SUMOylation of RanGAP1-C2 *in vitro*, for which only E1 and E2 (but not E3 ligase) are necessary, we hypothesized that either the E1 or E2 enzyme is the target of spectomycin B1. To test this idea, we first investigated whether spectomycin B1 directly binds to either E1 or E2. A pull-down assay using recombinant E1 or E2 proteins and spectomycin B1-conjugated agarose beads prepared by photoaffinity reaction[20] revealed that spectomycin B1 could directly bind to E2, but not E1 (Figure 3a). Surface plasmon resonance (SPR) analyses revealed that spectomycin B1 bound Ubc9 with a K_D value of 4.3 $\pm$ 1.1 μM and a 1:1 stoichiometry (Figure 3b). We next asked whether spectomycin B1 could block the formation of the E2-SUMO-1 intermediate without affecting the E1-SUMO-1 intermediate. The thioester-bond formation in the complex between either E1 or E2 and biotinylated SUMO-1 can be detected in the presence of ATP under nonreducing conditions using a biotin–avidin detection system.[21] The band intensity corresponding to the E2-biotinylated SUMO-1 intermediate was reduced by treatment with DTT, confirming that formation of the E2-SUMO-1 intermediate is sensitive to reducing agents. As anticipated, the amount of E2-SUMO-1 intermediate was greatly decreased by spectomycin B1 in a dose range similar to that required for inhibition of *in vitro* SUMOylation (Figure 3c). On the other hand, spectomycin B1 did not affect the E1-biotinylated SUMO-1 intermediate formation at 10 μM (Figure 3d), a concentration sufficient to inhibit both *in vitro* SUMOylation (Figure 1c) and formation of the E2-SUMO-1 intermediate (Figure 3c). By clear contrast, ginkgolic acid prominently inhibited formation of the E1-SUMO-1 intermediate formation under the same conditions, as previously reported (Figure 3d).[14] To further examine the specificity of spectomycin B1, we tested whether the compound inhibits the formation of intermediates between ubiquitin E2 enzymes and ubiquitin. All the ubiquitin E2 tested could form the intermediate in the presence of ATP under nonreducing conditions; the band corresponding to the E2-biotinylated ubiquitin intermediate disappeared when DTT was added to the reaction mixture (Figure 3e and SI Figure S3). Intermediate formation by any of the ubiquitin E2 enzymes was not affected by spectomycin B1 at 10 μM (Figure 3e and SI Figure S3), a concentration that was sufficient to inhibit E2-SUMO-1 intermediate formation (Figure 3c). Taken together, these observations suggest that spectomycin B1 selectively blocks formation of the E2-SUMO-1 thioester bond by directly binding to E2.

Antagonizing Ubc9 function in MCF7 breast-cancer cells inhibits ER-regulated gene expression, induces apoptosis, and reduces tumor volume in transplanted nude mice.[5,11] These observations prompted us to investigate whether spectomycin B1 exhibits antitumor activity against breast-cancer cells. First, we examined the effect of spectomycin B1 on the expression of pS2, one of the primary estrogen-response genes and a representative marker for ER-regulated genes.[22] The mRNA level of *pS2*, but not those of *Ubc9* and *Uba2*, was decreased in spectomycin B1-treated MCF7 cells (Figure 4a). Importantly, spectomycin B1 suppressed β-estradiol-dependent proliferation of MCF7 cells in a dose-dependent manner (Figure 4b). As anticipated, Ubc9 knockdown using siRNA oligos decreased β-estradiol-dependent proliferation of MCF7 cells (Figure 4c,d). This cell-growth inhibition by spectomycin B1 is mediated, at least in part, by inhibition of cellular Ubc9 activity, because Ubc9 overexpression counteracted the inhibitory effect of spectomycin B1 (Figure 4e,f). These results suggest that inhibition of SUMOylation by spectomycin B1 suppresses growth of breast-cancer cells by inhibiting ER signaling.

In this study, we discovered that spectomycin B1, previously known only as an antibiotic, is the first identified Ubc9 inhibitor. Spectomycin B1, together with its related metabolites, spectomycin A1 and A2, is a member of the aureolic acid class of antibiotics isolated from *Streptomyces spectabilis*.[16] These microbial metabolites contain dihydroxytetrahydroanthrocenone as common backbone architectures, although spectomycin B1 exists as a symmetric dimeric form (Figure 1a), whereas spectomycin A1 and A2 are monomeric forms.[16] In addition, we found that two structurally related natural products that occur as dimeric forms, chaetochromin A and viomellein (Figure 1a) also inhibit SUMOylation with *in vitro* IC_{50} values similar to that of spectomycin B1 (Figure 1 and SI Figure S2). Because these natural products share dihydroxytetrahydroanthrocenone as a common skeleton in their structures, we speculate that the dihydroxytetrahydroanthrocenone in spectomycin B1 is responsible for its ability to inhibit SUMOylation. Elucidation of the molecular mechanism by which spectomycin B1 and its related natural products inhibit protein SUMOylation will provide a basis for the design and development of more potent and specific Ubc9 inhibitors.

It has been suggested that disruption of SUMO homeostasis facilitates cancer development and progression;[9] therefore, enzymes that regulate protein SUMOylation in cells are regarded as attractive targets for cancer therapy. In the case of breast cancer, Ubc9 has been proposed as a target for anticancer drug development, because the mRNA level of *Ubc9* is increased in tumors[9] and inhibiting Ubc9 function prevents tumorigenesis.[5] Consistent with this hypothesis, this study revealed that spectomycin B1, which directly binds and inhibits Ubc9, reduces ER-dependent gene expression and suppresses proliferation of breast-cancer cells (Figure 4), although we cannot rule out the possibility that spectomycin B1 interacts with proteins other than Ubc9. However, it is still unclear how Ubc9 inhibition prevents hormone-dependent proliferation of breast-cancer cells. A number of oncogenic and tumor-suppressor proteins undergo SUMOylation.[2,3] Indeed, ERα transcriptional activity might be regulated through SUMO conjugation with ERα itself, or its coactivators or corepressors, which may be involved in ERα-dependent breast-cancer proliferation.[23] Thus, spectomycin B1 and its related natural products will serve as not only the starting materials for developing therapeutically effective Ubc9 inhibitors but also important tools for elucidating the molecular mechanisms by which Ubc9 acts in breast tumorigenesis.

METHODS

Plasmids and Antibodies. Plasmids for His-tagged SUMO-1, His and T7-tagged RanGAP1-C2, GST-Aos1/Uba2, His-tagged Ubc9, wild-type p53, p53 mutant (p53-K386R), and biotinylated SUMO-1 were previously described.[14] The plasmid containing cDNA for SUMO-1 was subcloned into the XhoI and *Pst*I sites of vector pEGFP-C1. Antibodies for SUMO-1 (N-19), Ubc9 (N-15), c-myc (9E10), and p53 (FL393-G) were purchased from Santa Cruz Biotechnologies. Antibodies for T7 and p53 (ab-6) were from Novagen and Calbiochem, respectively. Antibodies forα-tubulin (B-5−1−2) and FLAG (M2) were purchased from Sigma. Antibodies for GST (#3818−1) and GFP were obtained from Clontech.

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.[14]

***In Vitro* SUMOylation Assay.** The *in vitro* SUMOylation reaction was performed as described.[14]

Thioester Bond-Formation Assay. The reaction for the thioester bond formation between SUMO and E1 was performed as described.[14,21] For ubiquitin and ubiquitin E2 experiments, thioester bonds were analyzed with an ubiquitination kit (Enzo Life Sciences, Farmingdale, NY) following the protocols recommended by the manufacturer.

Cell Culture and Transfection. 293T and H1299 cells were maintained in Dulbecco's modified Eagle medium (DMEM) and MCF7 cells were cultured in RPMI1640 supplemented with 10% FBS at 37 °C in 5% CO2. Cells were seeded on 6-well dishes and grown to ~60% confluence at the time of transfection. Appropriate amounts of plasmid DNA were used for transfection, using Lipofectamine 2000 or Lipofectamine LTX with Plus reagent (Invitrogen).

Ubc9 Binding Assay. Spectomycin B1-immobilized beads were prepared as described.[20] The beads were incubated with either of GST-Aos1/Uba2 (E1) or His-tagged Ubc9 (E2) in the binding buffer (50 mM Tris [pH 7.6], 6 mM MgCl2, 150 mM NaCl, 0.1% Triton X-100) for 1 h at 30 °C. Beads were washed three times with binding buffer, and the bound proteins were eluted by boiling in SDS-PAGE sample buffer, resolved by electrophoresis, and analyzed by immunoblotting.

SPR Analysis. The SPR experiment was performed with a Biacore T200 (GE Healthcare). One μg/mL His-Ubc9, in 10 mM acetate buffer (pH 5.0, containing 6 mM $MgCl_2$), was immobilized to approximately 90 RU on a Series S Sensor Chip CM5 (GE Healthcare) using a standard amine coupling protocol with an Amine Coupling Kit (GE Healthcare). Analyte solution was prepared by a serial dilution of spectomycin B1 with buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 6 mM $MgCl_2$, and 0.05% Tween 20) containing 5% DMSO. Binding analysis was conducted at a flow rate of 30 μL/min at 25 °C. In each run, the association phase and the following dissociation phase were monitored for 60 and 120 s, respectively. From the obtained reference-subtracted sensorgrams, the dissociation constant (K_d) of spectomycin B1 was estimated by a global fitting to a simple 1:1 binding model in the Biacore evaluation software (GE Healthcare).

RT-qPCR. Total RNA was isolated using the RNeasy mini Kit (QIAGEN) and then reverse-transcribed using the RNA PCR Kit (AMV) (TaKaRa). cDNA fragments were analyzed by quantitative PCR using SYBR Premix EX *Taq*II (TaKaRa) and a LightCycler480 (Roche). The mRNA levels of all genes were normalized against β-actin as an internal control.

Cell-Proliferation Assay. MCF7 cells were seeded in duplicate onto 24-well dishes at a density of 15 000 cells/well. Cells were then treated with various concentrations of spectomycin B1 and β-estradiol for 96 h, using 10% charcoal-stripped serum-containing phenol red-free medium for culture. The cell number was counted using a Countess Cell Counter (Invitrogen).

ASSOCIATED CONTENT

Supporting Information

Oligonucleotides used in this work and additional figures and methods as described in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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