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Glaziovianin A Prevents Endosome Maturation *via* Inhibiting Microtubule Dynamics

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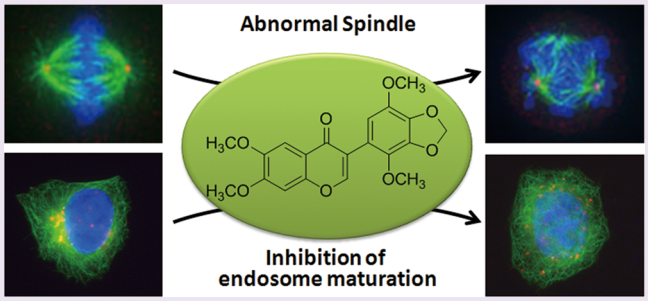
Supporting Information

ABSTRACT: Glaziovianin A, an isoflavone isolated from the leaves of *Ateleia glazioviana*, inhibits the cell cycle progression in M-phase with an abnormal spindle structure, but its inhibitory mechanism has not been revealed. Here, we report that glaziovianin A and its derivatives are microtubule dynamics inhibitors. Glaziovianin A extended the time lag of tubulin polymerization without changing the net amount of polymerized tubulin *in vitro* and suppressed microtubule dynamics in cells. Furthermore, glaziovianin A inhibited the transport of endosomes containing EGF-stimulated EGFR and prolonged the EGFR activation. Consistent with the prolonged activation of EGFR, glaziovianin A enhanced the EGF-dependent apoptosis in A431 cells. These results strongly suggested that microtubule dynamics is important for endosome transport and maturation, and that glaziovianin A shows cytotoxicity by two pathways, the mitotic arrest and inadequate activation of receptor kinases *via* the inhibition of endosome maturation.



Microtubules play important roles in mitosis, cell signaling, and motility in eukaryotes. Therefore, tubulin inhibitors have been recognized not only as antitumor agents but also as useful tools for understanding a variety of the cellular functions of microtubules. Two major classes of microtubule-targeting agents are used in the clinic, taxanes and *vinca* alkaloids, and these classes bind different sites of β-tubulin and show opposite effects *in vitro*. The other clinically used compounds, epothilones, also bind the taxane-binding sites on β-tubulin and stabilize microtubule filaments. However, these compounds sometimes cause severe side effects, including peripheral neuropathy, due to the extreme effects on microtubules of proliferating and nonproliferative cells. Because neuropathy is a common dose-limiting toxicity of these microtubule-targeting drugs, it is important to develop compounds without leading to the lesion.

Recently, it has been proposed that drugs with subtle effects on microtubules that only modulate microtubule dynamics may entail fewer side effects and be clinically useful.[1] One such compound is eribulin mesylate, a synthetic derivative of a marine sponge natural product, halicondrin B.[2] Eribulin mesylate decreases microtubule dynamics by binding to microtubules at the plus end.[3,4] The other example is noscapine.[5,6] However, noscapine is widely used as an antitussive medicine, noscapine and its analogues do not alter the steady-state polymer levels of tubulin, instead dampen microtubule dynamics enough to activate the mitotic checkpoints to halt mitosis without perturbing other vital microtubule functions. Therefore, microtubule dynamics inhibitors might be a novel type of antitumor agent, but it is important to examine whether there are unknown effects of these inhibitors on other cellular processes.

Endocytosis is the major pathway for internalization of membrane proteins from the cell surface. Ligand-stimulated growth factor receptors are incorporated into clathrin-coated vesicles and traverse the endocytic pathway by moving from clathrin-coated vesicles through early endosomes, late endosomes, and lysosomes for degradation. There are several reports that endocytosis is critical for the appropriate spatial localization of the ligand−receptor complex to activate downstream effectors,[7] and that the inhibition of EGFR endocytosis decreases the efficiency of signaling[8] and induction of

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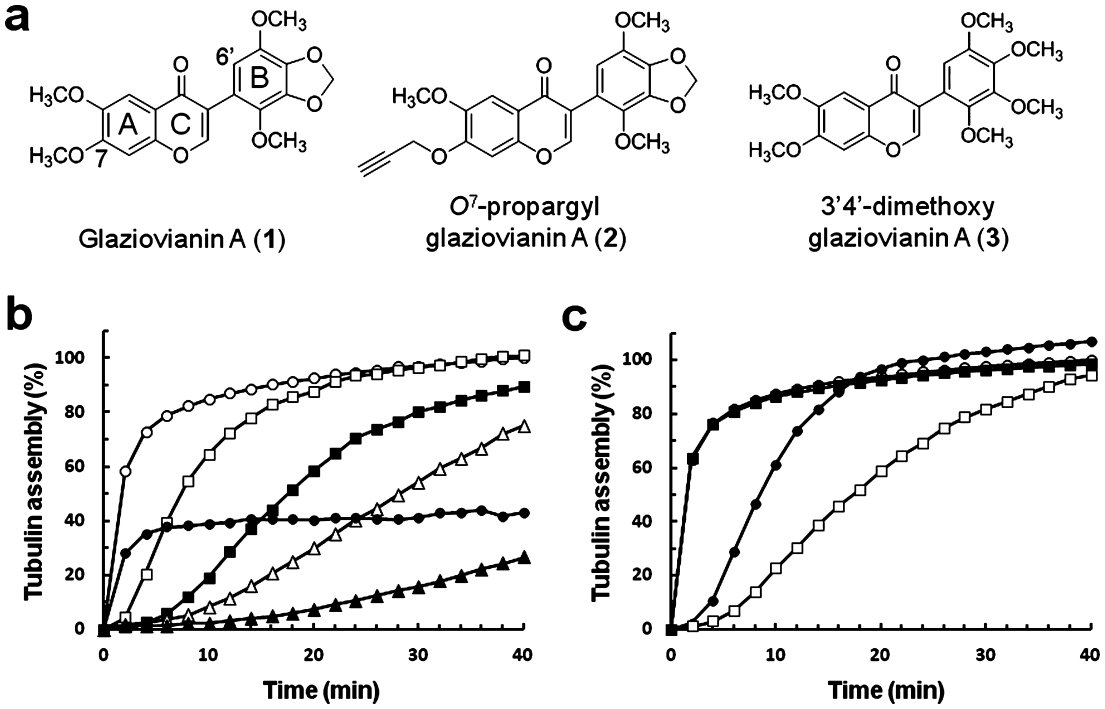

Figure 1. Glaziovianin A and its derivatives extended the time lag of microtubule polymerization *in vitro*. (a) Structure of glaziovianin A (**1**) and its derivatives (**2**, **3**). (b,c) Effects of glaziovianin A on tubulin polymerization *in vitro*. The final concentrations were 0 μM (○), 10 μM (□), 15 μM (■), 20 μM (△), and 23 μM (▲) of **1**, or 10 μM colchicine (●) in b, and 10 μM of compounds **1** (●), **2** (□), and **3** (■), or DMSO (○) in c.

apoptosis.[9,10] Recently, it was reported that the very low concentration of paclitaxel restricted the endocytic transport not solely by the rearrangement of microtubules,[11] suggesting that microtubules are not a simple highway for endosome. Furthermore, it was reported that EB1, one of the microtubule plus-end tracking proteins, binds Gapex-5.[12] Considering that Gapex-5 is a GEF protein for Rab5, a key regulator for early endosomes, microtubule dynamics might be important for the endosome maturation. However, the relationship between microtubule dynamics and endosome maturation has not been verified.

Glaziovianin A (Figure 1) is an isoflavone isolated from the leaves of *Ateleia glazioviana* (Legminosae) as a cytotoxic compound against human leukemia HL-60 cell.[13] Glaziovianin A displayed differential cytotoxicities in the Japanese Foundation for Cancer Research 39 cell line panel assay,[14] with the pattern of the differential cytotoxicities correlating to that exhibited by TZT-1027, a microtubule inhibitor, and glaziovianin A inhibited the cell cycle progression in M-phase with abnormal spindle structure. We analyzed the structure–activity relationships of glaziovianin A and succeeded in synthesizing derivatives that showed potent cytotoxic activity.[15,16] However, these molecular targets and inhibitory mechanisms have not been determined. Here, we report that glaziovianin A is a novel microtubule dynamics inhibitor, which decreases both elongation and shortening rates of microtubule and drastically increase pause populations in cells. The inhibition of microtubule dynamics by glaziovianin A resulted in not only mitotic arrest but also perturbation of endosome transport, which induced the prolonged EGFR phosphorylation and the EGF-mediated apoptosis. Furthermore, the same inhibition of endosome transport was observed in the cells treated with low concentrations of vinblastine and paclitaxel at which microtubule dynamics was impaired. These results suggested that microtubule dynamics is important for not only bipolar spindle formation but endosome transport and maturation, and that the microtubule dynamics inhibitors, including glaziovianin A, induce apoptosis by two pathways, mitotic arrest and accumulation of activated receptor kinases in early endosomes.

We reported that glaziovianin A and its derivatives inhibited the cell cycle progression in M-phase with abnormal spindle structure. which was different from those induced by colchicine and vinblastine, the classical microtubule inhibitors.[13,16] Recently, several studies have reported that microtubule dynamics inhibitors also induce mitotic arrest *via* induction of abnormal spindles.[17,18] Therefore, we investigated the effects of glaziovianin A on the microtubule polymerization *in vitro* (Figure 1b). Purified porcine tubulin was incubated with or without compounds at 37 °C and then polymerized by addition of glutamate. After a short time lag, tubulin started to polymerize and reached equilibrium in the DMSO control. Colchicine and vinblastine, potent inhibitors of microtubule polymerization, inhibited the polymerization and decreased the net amount of polymerized tubulin in a dose-dependent manner (Figure 1b and Supporting Figure S1). In contrast, glaziovianin A elongated the time lag in a dose-dependent manner but did not change the net amount of polymerized tubulin. The same results were obtained by using a potent derivative, O^7-propargyl (**2**), but not by using the inactive derivative 3′4′-dimethoxy glaziovianin A (**3**) (Figure 1c).[15] The competition binding experiments using [^{3}H]-labeled vinblastine and colchicine suggested that the binding site of glaziovianin A is identical or close to that of colchicine and noscapinoids (Supporting Table S1).[19]

We next observed the effects of glaziovianin A on the microtubule network in cells by immunofluorescence microscopy. HeLa cells treated with or without compounds were fixed

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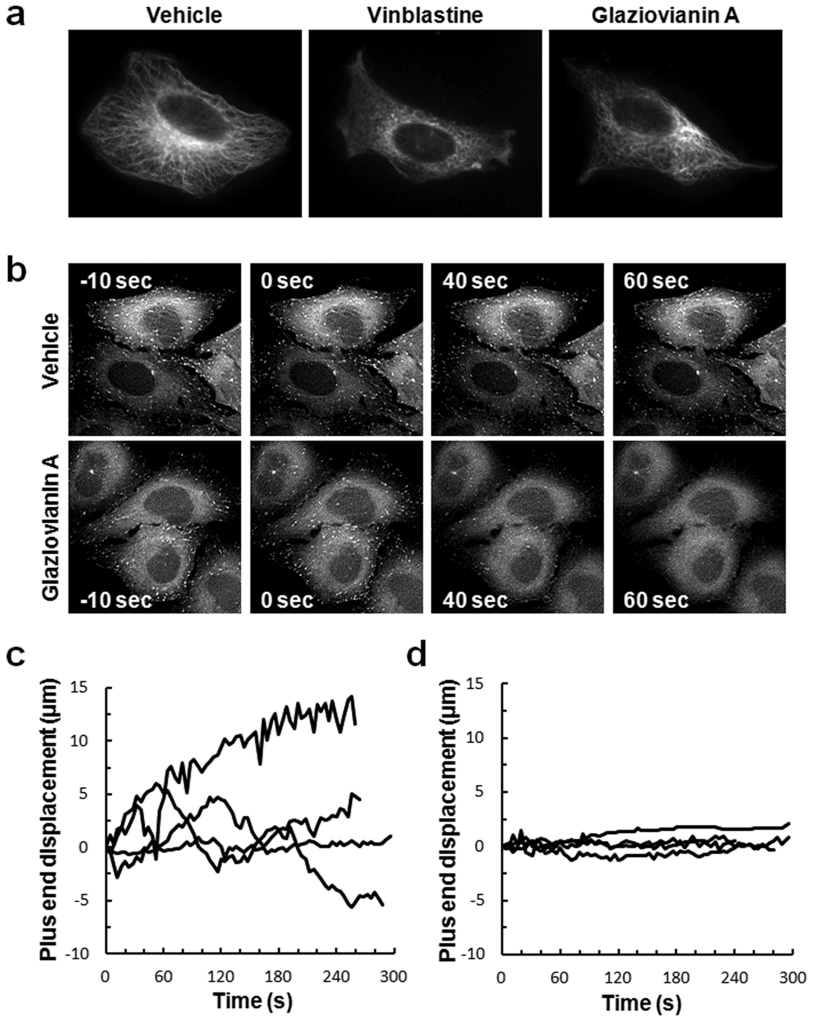

Figure 2. Glaziovianin A inhibited the microtubule dynamics in cells. (a) Effects of glaziovianin A on the microtubule network in interphase cells. HeLa cells were treated with glaziovianin A, vinblastine, or in the absence of either compound for 6 h. (b) Effects of glaziovianin A on the behavior of EB1. EB1-Venus-expressing HeLa cells were treated with glaziovianin A. Elapsed time is indicated. (c,d) Life history plots of microtubules in (c) DMSO- and (d) glaziovianin A-treated PtK2 cells. The positions of the microtubule ends were followed to describe the growth and shortening of the microtubule plus end. Each line represents a separate microtubule.

Table 1. Parameters of Microtubule Dynamics[a]

	growth (μm min^{-1})	shortening (μm min^{-1})	catastroph frequency (s^{-1})	rescue frequency (s^{-1})	time in growth %	time in pause %	time in shortening %
DMSO	11.2 ± 1.4	13.0 ± 1.1	0.055 ± 0.007	0.113 ± 0.011	37.3	27.3	35.5
glaziovianin A	5.3 ± 0.7*	6.2 ± 0.7*	0.024 ± 0.005*	0.119 ± 0.015	16.0	71.7	12.3

[a]The rates of growth and shortening were calculated from the slopes of the life history plots of 26 microtubules in five control cells and 26 microtubules in eight glaziovianin A-treated cells as described in the Methods. Values are the mean ± SE. *, $P \leq 0.01$.

and stained with anti-α-tubulin antibody. The microtubule network in interphase cells was completely disrupted by 10 nM vinblastine (Figure 2a). In contrast, glaziovianin A did not induce collapse of the microtubule networks in interphase cells even at a concentration (3 μM) sufficient to induce abnormal spindles in M phase cells.[15] To determine whether glaziovianin A decreased the polymerization rate in cells, the dynamic behavior of Venus-tagged EB1 at the microtubule plus ends was observed in live cells (Figure 2b and Supporting Movies S1 and S2). As previously reported, numerous Venus signals were found moving from the centrosome region to the cell periphery, indicating that EB1-Venus were concentrated at the plus ends of dynamically growing microtubules.[20] These concentrations of EB1-Venus became undetectable within 1 min of incubation with glaziovianin A, strongly suggesting that glaziovianin A inhibits plus-end growing of microtubules. To investigate whether glaziovianin A inhibits only microtubule growing or not, we next measured the microtubule dynamics parameters using PtK2 cells transiently expressing EGFP-α-tubulin (Figure 2c, Table 1, and Supporting Movies S3 and S4). Consistent with previous reports,[5] microtubules in control cells were highly dynamic. On the contrary, cells treated with glaziovianin A had microtubules with decreased growth and shrinkage rates and with low frequencies of catastrophe. The drastic increase in pause population was also observed (Table

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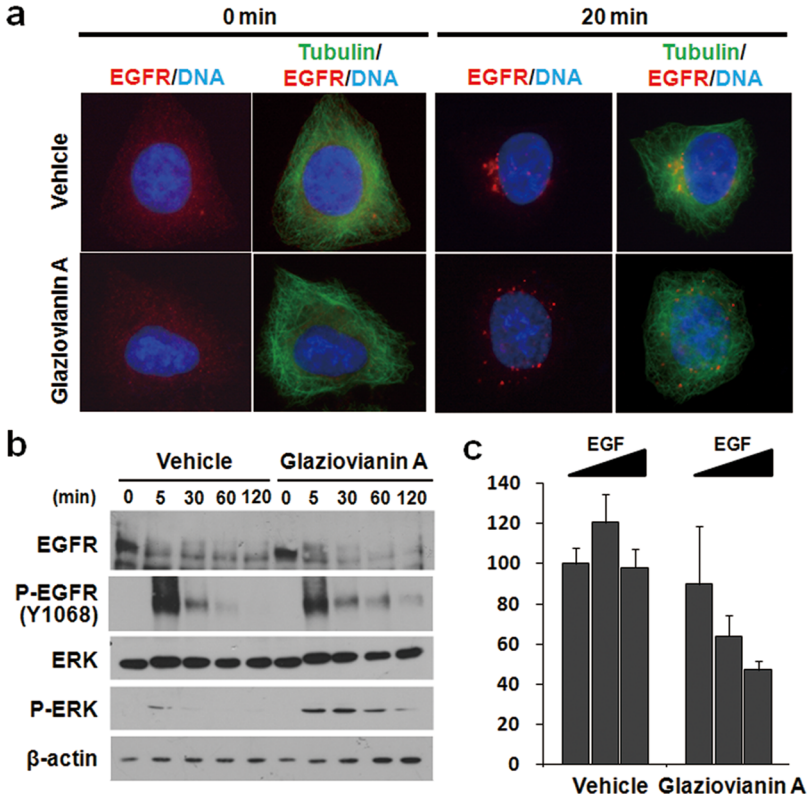

Figure 3. Glaziovianin A inhibited the downregulation of activated EGFR and induced cell death. (a) Effects of glaziovianin A on the EGF-stimulated endocytosis. Serum-starved HeLa cells were pretreated with glaziovianin A and then stimulated with EGF for 20 min. Microtubules (green), EGFR (red), and DNA (blue) were observed. (b) Glaziovianin A sustained EGF-stimulated phosphorylation of EGFR and ERK. Glaziovianin A-pretreated, serum-starved HeLa cells were stimulated with EGF and harvested at the indicated times. (c) Glaziovianin A induced the cell death of A431 in an EGF-dependent manner. Glaziovianin A-pretreated, serum-starved A431 cells were treated with various concentrations of EGF for 24 h.

1). These results strongly suggest that glaziovianin A suppresses microtubule dynamics in cells.

Recently, it has reported that EB1 binds Gapex-5, the GEF protein for Rab5.[12] Because Rab5 is a pivotal protein in the early step of endocytosis and the transport of EB1 to the cell periphery was inhibited by glaziovianin A (Figure 2b), we thought for the possibility that perturbation of the microtubule dynamics may have interfered with endocytosis, especially endosome maturation of early endosomes to late endosomes. To test this idea, we investigated the effects of glaziovianin A on the endocytosis of EGF-stimulated EGFR (Figure 3a). Serum-starved HeLa cells were pretreated with glaziovianin A for 1 h and stimulated by EGF. Before EGF stimulation, EGFR were distributed on the plasma membrane even in the presence of glaziovianin A (Figure 3a, 0 min). Immediately after stimulation with EGF, EGFR was efficiently incorporated and transported to the vicinity of the nuclei within 20 min in the absence of glaziovianin A. On the contrary, glaziovianin A drastically inhibited EGFR transport without disrupting the microtubule network. The same phenotype was observed in endocytosis of Alexa^{568}-labeled transferrin (Supporting Figure S2).

There are several reports that endocytosis is inhibited by disrupting the microtubule network.[21−23] To confirm whether the inhibition of endosome transportation is a specific event of glaziovianin A, we investigated the effects of vinblastine and paclitaxel at the low concentrations, which microtubule dynamics were inhibited but microtubule network was not disrupted. The low concentrations of vinblastine and paclitaxel also inhibited EGFR transport without affecting the microtubule network (Supporting Figure S3). Therefore, these results suggested that microtubule dynamics is important for the proper transport of endosomes.

It is commonly known that the receptor kinases on the plasma membrane are activated by ligand binding and incorporated into the endosome. During endosome maturation, receptor kinase activity is decreased by ligand dissociation and degradation in the endosome. Because EGF-dependent endocytosis was inhibited by glaziovianin A, it was thought that this compound also inhibits the downregulation of receptor kinase activity and signal transduction. To confirm this hypothesis, we investigated the effects of glaziovianin A on phosphorylation of EGFR and its downstream kinase, ERK. Serum-starved HeLa cells were treated with or without glaziovianin A for 1 h, and stimulated with EGF. The EGF-stimulated cells were harvested at the indicated time, and the phosphorylation levels of EGFR and ERK were measured by immunoblotting (Figure 3b). No phosphorylated EGFR was detected in the absence of serum, but EGFR was rapidly phosphorylated at 5 min after EGF-stimulation. This phosphorylation gradually decreased and became undetectable at 2 h after stimulation. ERK kinase was phosphorylated with the same kinetics as EGFR phosphorylation. In contrast, glaziovianin A sustained phosphorylation of EGFR and ERK for more than 2 h. Because glaziovianin A did not influence the EGF-binding to EGFR (Supporting Figure S4), these results suggested that glaziovianin A inhibits the downregulation of growth factor signal transduction.

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There are several reports that the inhibition of endosome acidification by bafilomycin A and monensin induces EGF-dependent apoptosis[10,24] or growth arrest,[25] probably by prolonging EGFR activation in EGFR-overexpressing cells. Because EGFR and ERK activation were sustained more than 2 h by glaziovianin A treatment, we speculated that glaziovianin A also induced EGF-dependent cell death. To investigate this possibility, we performed a cell viability assay using a combination of glaziovianin A and EGF in the EGFR-overexpressing cell line A431 (Figure 3c). Serum-starved A431 cells were treated with 2 μM glaziovianin A for 1 h and stimulated with EGF for 24 h. In the absence of glaziovianin A, no obvious cytotoxicity was observed, even in the presence of a high concentration of EGF, suggesting that EGF itself does not induce cell death in A431 cells. In contrast, in the presence of glaziovianin A, EGF drastically decreased the cell viability in a dose-dependent manner (Figure 3c). These results clearly show that glaziovianin A induced EGF-dependent cell death in A431 cells. Because, unlike V-ATPase inhibitors, glaziovianin A did not inhibit the acidification of intracellular acidic organelles at all (data not shown), these results suggest that glaziovianin A exerts its cytotoxic effects by sustaining the intracellular signals generated from the EGF/EGFR complex, but not through the inhibition of organelle acidification.

In summary, glaziovianin A is a novel microtubule dynamics inhibitor and shows antitumor activities by two apoptotic pathways: the mitotic arrest induced by activating the spindle checkpoint and prolongation of the sustaining growth signal from the EGF/EGFR complex. Our results suggest that the microtubule dynamics is important not only for mitotic spindle formation but also for the regulation of endosome maturation.

METHODS

Reagents and Antibodies. Glaziovianin A and its derivatives were synthesized as described.[15,16] Other chemicals were purchased from Wako and dissolved in DMSO. Human recombinant EGF was purchased from Research & Diagnostics Systems Inc. Antibodies specific for EGFR (#sc-03) and α-tubulin (#sc-32293) were purchased from Santa Cruz Biotechnology. Antibodies for p-EGFR (Y1068, #3777) and anti-β-actin (#ab8227-50) were from Cell Signaling Technology and Abcam, respectively.

Tubulin Purification and Polymerization *in Vitro*. Tubulin was purified from porcine brain using high-molarity buffer.[26] Tubulin polymerization assay was performed as reported.[27]

Cell Culture and Immunofluorescence Procedure. HeLa, PtK2, and A431 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) FCS in a humidified atmosphere containing 5% CO_2.

For determination of the EGFR localization, serum-starved HeLa cells were treated with 3 μM glaziovianin A or 10 nM vinblastine for 1 h, and stimulated with 100 ng/mL EGF for 20 min. Cells were fixed with −20 °C MeOH and stained.

Live Cell Microscopy. Timelapse analysis of EB1-Venus and EGFP-α-tubulin were performed on a microscope controlled by DeltaVision SoftWorx (Applied Precision) using a ×60/1.40 oil immersion objective at 37 °C. EB1-Venus HeLa cells were treated with 3 μM glaziovianin A, and Venus signals were captured at 2 s intervals.[28] PtK2 cells expressing EGFP-α-tubulin were treated with 1 μM glaziovianin A, and 3 sections with 0.4 μm intervals were collected every 4 s.[29] The positions of the microtubule ends were followed using ImageJ software and graphed as life history plots.[5] The rates of growth and shortening were calculated from the slopes of the plots using linear regression. If the change in length of a microtubule between two successive time points was greater than 0.5 μm, it was considered real growth or shortening. Catastrophe was defined as the transition from either growth or pause to shortening, and the catastrophe frequency was calculated as the number of transitions divided by the time spent in growth and pause. Rescue was defined as the transition from shortening to either growth or pause, and the rescue frequency was calculated as the number of such events divided by the time spent shortening.

EGF Stimulation, Immunoblot Analysis, and WST-8 Procedures. Serum-starved HeLa cells were treated with 3 μM glaziovianin A for 1 h and then with 10 ng/mL EGF for 5−120 min.

Serum-starved A431 cells were treated with or without 2 μM glaziovianin A for 1 h and then treated with 0, 30, and 100 ng/mL of EGF for 24 h. Cell growth was determined using a cell counting kit-8 (Dojindo).

ASSOCIATED CONTENT

Supporting Information

Supplementary methods, movies, and figures. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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[¶]These authors contributed equally to this work.

Notes

The authors declare no competing financial interest.

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