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Cucurbitacin E as a new inhibitor of cofilin phosphorylation in human leukemia U937 cells

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ABSTRACT

Cucurbitane-type triterpenes, cucurbitacins B and E, were reported to exhibit cytotoxic effects in several cell lines mediated by JAK/STAT3 signaling. However, neither compound inhibited phosphorylation of STAT3 in human leukemia (U937) cells at low concentrations. We therefore synthesized a biotin-linked cucurbitacin E to isolate target proteins based on affinity for the molecule. As a result, cofilin, which regulates the depolymerization of actin, was isolated and suggested to be a target. Cucurbitacins E and I inhibited the phosphorylation of cofilin in a concentration-dependent manner, and their effective concentrations having the same range as the concentrations at which they had cytotoxic effects in U937 cells. In addition, the fibrous-/globular-actin ratio was decreased after treatment with cucurbitacin E in HT1080 cells. These findings suggested that the inhibition of cofilin's phosphorylation increased the severing activity of cofilin, and then the depolymerization of actin was enhanced after treatment with cucurbitacin E at lower concentrations.

Apoptosis plays an important role in the maintenance of tissue homeostasis through the selective elimination of excessive cells. On the other hand, induction of apoptosis of cancer cells is also useful for treatment of cancer, since the cytotoxic drugs (e.g., etoposide, cisplatin, and paclitaxel) used in chemotherapy cause apoptosis in target cells.[1–4]

Previously, cucurbitacins B, E (**1**), and I (**2**) and several related compounds were reported to show cytotoxic effects in several cell lines such as A549, MDA-MB-468, HepG2, and KB, and their apoptosis-inducing activities mediated by the inhibition of Janus kinase (JAK)/signal transducer and activator of transcription 3 (STAT3) signaling.[5–10] We have recently reported that cucurbitacins B and E (**1**) isolated from the roots of *Bryonia cretica* showed strong cytotoxic effects in human leukemia (U937) cells, and an α,β-conjugated ketone moiety at the 22–24-positions and an acetoxy group at the 25-position are important for the greater activity.[11]

In the present study, we compared effects of the active triterpene, cucurbitacin E (**1**),[12,13] and a moderate active triterpene, cucurbitacin I (**2**),[14,15] on proliferation of U937, human leukemia (HL60), and human fibrosarcoma (HT1080) cells (Fig. 1).[16] Compound **1** showed greater activity than **2**; IC_{50} values of **1** were 16, 18, and 40 nM, and those of **2** were 0.30, 0.10 and 0.47 μM against U937, HL60, and HT1080 cells, respectively, after incubation for 72 h. These findings confirm the importance of an acetoxy group at the 25-position.[11,17]

With regard to effects on the cell cycle, inhibitors of JAK/STAT3 signaling are reported to induce arrest at G_0/G_1 in several human and mouse cancer cell lines.[18–20] However, few effects of cucurbitan-type triterpenes on cell cycle arrest have been reported to date, though a weaker active 23,24-dihydrocucurbitacin B was reported to induce arrest at G_2/M in human breast cancer cells (Bcap37).[21] Therefore, we examined the effect of cucurbitacin E (**1**) on the cell cycle in U937 cells by flow cytometry after staining with propidium iodide (PI).[22]

Consistent with a previous report on 23,24-dihydrocucurbitacin B,[21] the population of cells in the G_0/G_1 and S phases was reduced [G_0/G_1 (control: 45.1%, **1**: 37.7%), S (control: 44.0%, **1**: 28.1%)] and that in G_2/M was increased (control: 10.9%, **1**: 34.2%) by treatment with **1** (100 nM) for 24 h, indicating that **1** caused arrest at G_2/M. Furthermore, **1** did not inhibit the phosphorylation of STAT3 at concentration 10 μM, and inhibit it at a higher concentration (10 μM) in U937 cells (Fig. 2).[23] Similar results were observed after treatment with cucurbitacin B (data not shown). Since our results are not consistent with reports about an inhibitor of STAT3 phosphorylation, other mechanisms of action of cucurbitane-type triterpenes were considered to exist.

Next, we synthesized a biotin-linked cucurbitacin E (**3**)[24,25] and tried to isolate target proteins based on its affinity for the

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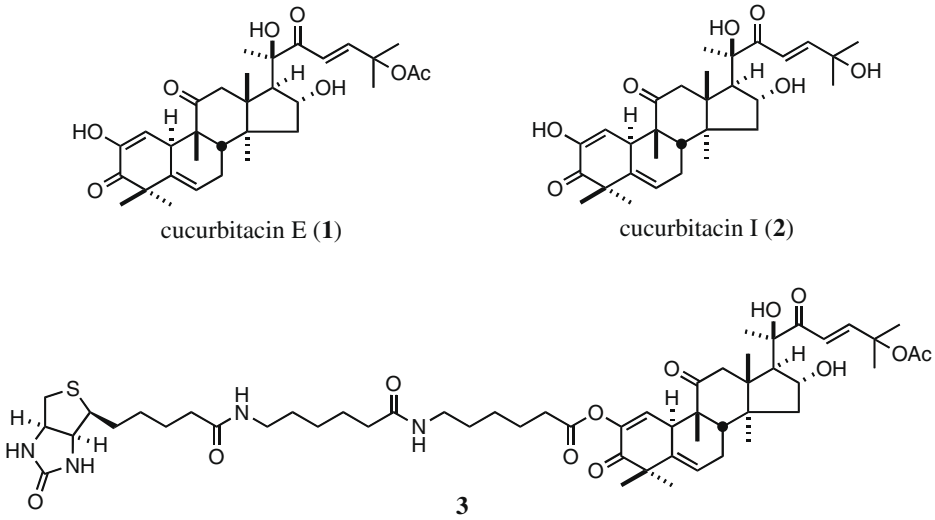

Figure 1. Chemical structures of cucurbitacins E (**1**) and I (**2**) and biotin-linked cucurbitacin E (**3**).

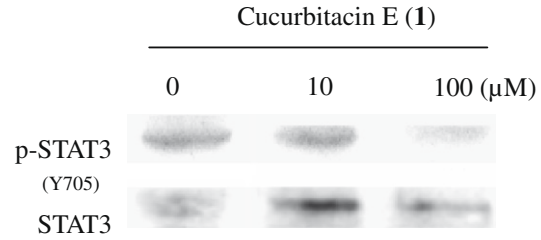

Figure 2. Effects of cucurbitacin E (**1**) on phosphorylation of STAT3 in U937 cells. U937 cells (5.0×10^4 cells/mL) were incubated with the test compound for 24 h. Equivalent amount of protein (60 μg of protein/lane) of each lysate was electrophoresed in 10% SDS–polyacrylamide gels. The STAT3 and p-STAT3 proteins were detected using Western blotting.[23]

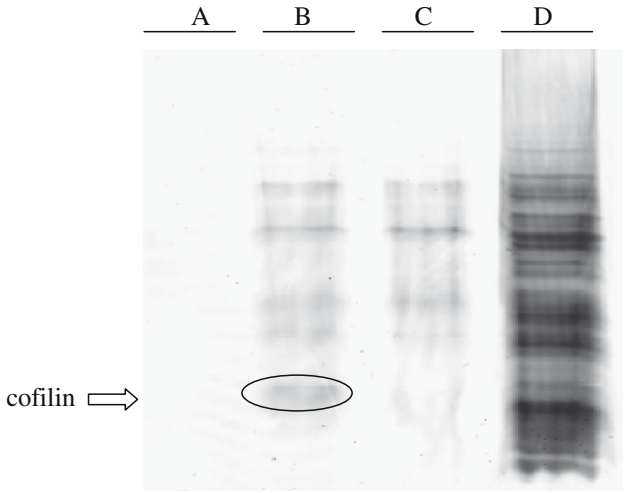

Figure 3. Isolation of cucurbitacin E (**1**)-selective binding protein from U937 cells. A: cucurbitacin E (**1**, 1 μM), B: biotin-linked cucurbitacin E (**3**, 1 μM), C: biotin-linked cucurbitacin E (**3**, 1 μM) + cucurbitacin E (**1**, 100 μM), D: total protein. U937 cells were harvested from a 50 mL culture (about 5.0×10^7 cells), resuspended in 500 μL of a lysis buffer (pH 7.0, 50 mM imidazole, 50 mM NaCl, 5 mM 6-aminohexanoicacid, 0.5% DDM, 1.1% triton-X100, proteinase inhibitor, and phosphatase inhibitor), and sonicated for 90 s three times on ice. Debris was removed by centrifugation of the sample at 10000*g* for 10 min. Test compounds were incubated with 500 μL of the cell lysates for 30 min on ice in binding buffer (50 mM ammonium carbonate and 0.5 M NaCl), and the mixture was passed through HiTrap Streptavidin HP (GE Healthcare). After washing with the binding buffer, the bound proteins were eluted with the elution buffer (50 mM ammonium acetate and 0.5 M NaCl) according to the manufacturer's instructions and separated by 10% SDS–PAGE. The 20 kDa band specific to the test compound was excised from the gel. After in-gel digestion, tryptic peptides were subjected to LC–MS/MS analysis.[26]

molecule. Briefly, cell lysate without SDS was treated with **3** and passed through a streptavidin-column. After washing with a washing buffer, bound proteins were eluted with an elution buffer and separated by SDS–PAGE (Fig. 3). A band at 20 kDa that was excluded by addition of a high concentration of **1**, considered specific to **1**, was excised from the gel. After in-gel digestion, tryptic peptides were subject to LC–MS/MS analysis.[26] As a result, cofilin was isolated and suggested to be a target protein. Cofilin is a member of a family of essential conserved small actin-binding proteins that play pivotal roles in cytokinesis, endocytosis, embryonic development, stress response, and tissue regeneration.[27] In response to stimuli, cofilin promotes the regeneration of actin fila-

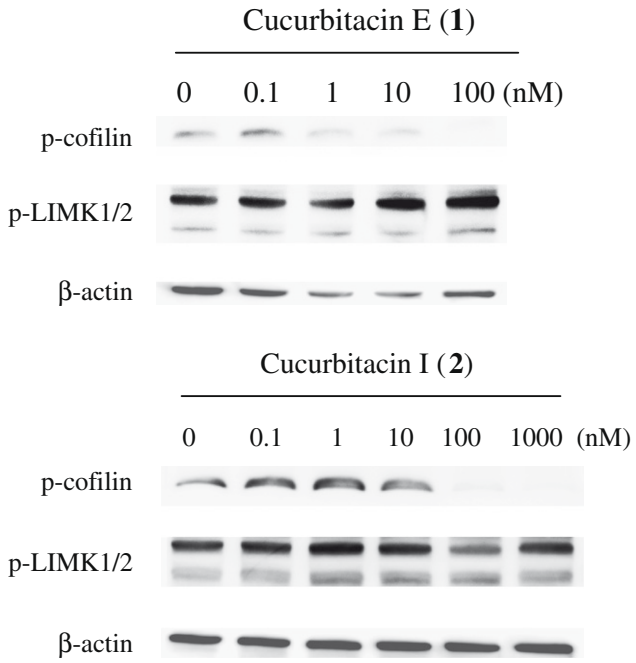

Figure 4. Effects of cucurbitacins E (**1**) and I (**2**) on p-cofilin and p-LIMK1/2 levels in U937 cells. U937 cells (5.0×10^4 cells/mL) were incubated with the test compounds for 24 h. Equivalent amount of protein [60 μg of protein/lane for phospho-cofilin (p-cofilin) and phospho-LIMK1/2 (p-LIMK1/2), 30 μg of protein/lane for β-actin] of each lysate was electrophoresed in 10% SDS–polyacrylamide gels. The p-cofilin, p-LIMK1/2, and β-actin proteins were detected using Western blotting.[23]

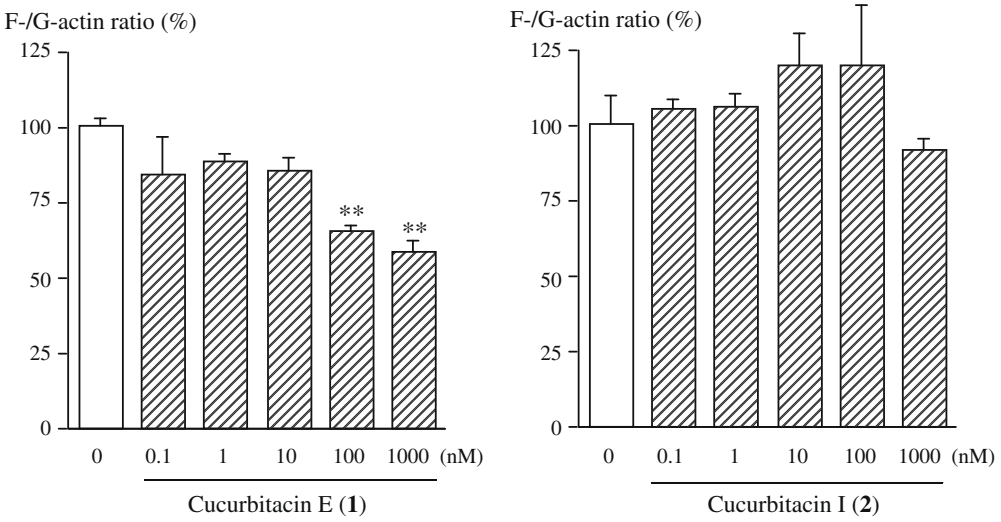

Figure 5. Effects of cucurbitacins E (**1**) and I (**2**) on F-/G-actin ratio in HT1080 cells. HT1080 cells (1.0×10^4 cells/100 μL/well) were seeded in 96-well microplates and incubated with test compounds for 24 h at 37 °C. The cells were then washed three times with PBS, and fixed with 4% formaldehyde in PBS for 15 min. Next they were permeabilized with ice-cold methanol and blocked with Blocking One for 30 min. After washes with PBS, the cells were incubated for 15 min at rt in the dark with the Alexa Fluor® 350 phalloidin (Invitrogen) in PBS. The cells were then incubated for 15 min at rt in the dark with the deoxyribonuclease Alexa Fluor® 488 conjugate (Invitrogen) in PBS. After another washes with PBS, the fluorescence intensity of each well was measured with a microplate reader (ex: 355 nm, em: 405 nm and ex: 480 nm, em: 520 nm, FLUOstar OPTIMA, BMG Labtechnologies). Each bar represents the mean with SEM ($n = 4$). Significantly different from the control group, $^{**}p < 0.01$ (Dunnett's test).

ments by severing preexisting filaments.[28] The severing activity of cofilin is suppressed by its phosphorylation by LIM kinase (LIMK) or TES kinase.[29–31]

Next, to confirm the involvement of cofilin in the cytotoxic effect of cucurbitane-type triterpenes, effects of cucurbitacins E (**1**) and I (**2**) on the phosphorylation of cofilin were examined using a SDS–PAGE and Western blotting.[22] As shown in Figure 4, both compounds inhibited the phosphorylation of cofilin, but not LIMK1/2, in a concentration-dependent manner. In addition, the range of concentrations at which they were effective was the same as that at which they were cytotoxic in U937 cells.

Finally, we examined the fibrous (F)-/globular (G)-actin ratio to examine the influence of cucurbitacins E (**1**) and I (**2**) on the structure of actin in an adherent cell line, HT1080 cells, using fluorescent probes [Alexa Fluor® 350 phalloidin and deoxyribonuclease Alexa Fluor® 488 (Invitrogen)] for F- and G-actins. The F-/G-actin ratio was decreased after treatment with cucurbitacin E (**1**) at 100 nM. Cucurbitacin I (**2**) tended to decrease the ratio at 1000 nM, but not significant. These findings suggested that cofilin's severing activity increased when its phosphorylation was inhibited and the depolymerization of actin was enhanced after treatment with **1** at low concentrations, with **1** eventually exhibiting antiproliferative effect. Compound **2** also tended to decrease the F-/G-actin ratio at 1000 nM, though the decrease was small. This result implies that the inhibition of JAK/STAT3 signaling is also important at higher concentrations as reported (Fig. 5).[8–10] To the best of our knowledge, this is the first report about a low molecular inhibitor against cofilin's phosphorylation at low concentrations.

In conclusion, we synthesized a biotin-linked cucurbitacin E (**1**) and tried to identify target proteins based on its affinity. As a result, cofilin, which regulates the depolymerization of actin, was isolated. Cucurbitacins E (**1**) and I (**2**) inhibited the phosphorylation of cofilin in a concentration-dependent manner, and the range of concentration at which they were effective was the same as that at which they were cytotoxic in U937 cells. In addition, F-/G-actin ratio was decreased after treatment with cucurbitacin E (**1**) in HT1080 cells. These findings suggested that the inhibition of cofilin's phosphorylation increased the severing activity of cofilin, and then the depolymerization of actin was enhanced after treatment with **1** at lower concentrations.

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16. U937 cells (Cell No. JCRB9021), HL60 cells (Cell No. JCRB0163), and HT1080 cells (Cell No. JCRB9113) were obtained from Health Science Research Resources Bank (Osaka, Japan), and the cells were grown in RPMI-1640 and MEM supplemented with 10% FBS, penicillin (100 units/mL), and streptomycin (100 μg/mL) at 37 °C in 5% CO_2/air. After 68 h incubation of U937 cells (5.0×10^3 cells/100 μL/well), HL60 cells cells (5.0×10^3 cells/100 μL/well), or HT1080 cells (5.0×10^3 cells/100 μL/well), with test compounds in RPMI-1640 in 96-well microplates, 10 μL of WST-8 solution (Cell Counting Kit-8™, Dojin)

was added to each well. After a further 4 h in culture, the optical density of the water-soluble formazan produced by the cells was measured with a microplate reader (Model 550, Bio-Rad) at 450 nm (reference: 655 nm). Inhibition (%) was calculated and IC_{50} value was determined graphically.

17. The IC_{50} values of cucurbitacins B and D and 23,24-dihydrocucubitacins B and E were 13 nM, 0.28 μM, 0.29 μM, and 3.1 μM in HL60 cells and 27 nM, 0.50 μM, 1.2 μM, and 9.6 μM in HT1080 cells, respectively. These results confirm the importance of a double bond at the 23,24-positions as well as an acetoxy group at the 25-position.
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22. After a 24 h incubation of U937 cells (5.0×10^4 cells/mL) with test compounds, cells were harvested, transferred to test tubes and centrifuged at 1500 rpm for 5 min at 4 °C. The supernatant was discarded and the cell pellets were resuspended in 300 μL of cold PBS and fixed by the addition of 700 μL of ice-cold ethanol. Fixed cells were incubated overnight at −20 °C after which they were centrifuged at 1500 rpm for 5 min. The cell pellets were resuspended in 500 μL of PBS with DNase-free RNase (0.25 mg/mL) and incubated in the dark at 37 °C for 30 min. After the incubation, propidium iodide (final concn: 50 μg/mL) was added. The flow cytometric determination of DNA content (20,000 cells/sample) was analyzed with a FACSCalibur Flow Cytometer (Becton Dickinson). The fractions of cells in G_0/G_1, S, and G_2/M phase were analyzed using cell cycle analysis software, ModFit LT ver. 3.0 (Verity Software House).
23. U937 cells (5.0×10^4 cells/mL) were incubated with the test compounds for 24 h. They were then lysed in a lysis buffer containing a phosphatase inhibitor (Roche) and a proteinase inhibitor (Thermo Scientific). Protein concentrations of cell lysate were determined using the Protein Assay Kit (Thermo Scientific). Equivalent amounts of protein (60 μg or 30 μg of protein/lane) were electrophoresed in 10% SDS–polyacrylamide gels and transferred to polyvinylidene difluoride membranes. The membranes were blocked for 30 min in Blocking One or Blocking One-P (Nacalai Tesque). The blots were probed with primary antibody at the appropriate dilution in Tris-buffered saline containing 0.1% Tween 20 (T-TBS) for 1 h. The membranes were then washed three times with T-TBS and incubated with the appropriate HRP-conjugated secondary antibody for 1 h. Immunoreactive proteins were detected using an enhanced chemiluminescence kit (ECL plus, GE Healthcare), according to the manufacturer's instructions. The following antibodies and dilutions were used for Western blotting: rabbit polyclonal antibody against anti-phospho-STAT3, anti-STAT3, anti-phospho-cofilin, anti-phospho-LIMK1/2, anti-β-actin (1:1000) (Cell Signaling Technology); anti-rabbit IgG; and HRP-linked whole antibody from donkeys (1:5000) (GE Healthcare).
24. A solution of D-biotinylaminohexanoylaminohexanoic acid[25] (24 mg, 0.05 mmol) in pyridine (0.5 mL) was treated with **1** (36 mg, 0.065 mmol) in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC·HCl, 19 mg, 0.10 mmol) and 4-dimethylaminopyridine (4-DMAP, 12 mg, 0.10 mmol) and the solution was stirred under N_2 at room temperature for 49 h. The solvent was removed under reduced pressure and the residue was purified by preparative TLC [PTLC, $CHCl_3$/MeOH (10:1, v/v)] to give **3** (23 mg, 45%). The methods of synthesis will be presented in detail in a full paper.
3: a white powder. $[\alpha]_D^{17}$ −21.9 (*c* 0.20, MeOH). HR-FAB-MS *m*/*z*: Found, 1009.5566 $[M+H]^+$ (Calcd for $C_{54}H_{81}N_4O_{12}S$: 1009.5572), Found, 1031.5396 $[M+Na]^+$ (Calcd for $C_{54}H_{80}N_4O_{12}SNa$: 1031.5391). IR (KBr): 3440, 3300, 2999, 2936, 1697, 1655, 1259, 1232 cm^{-1}. ^1H NMR (270 MHz, $CDCl_3$) δ: 0.99, 1.03, 1.31, 1.31, 1.39, 1.44 (3H each, all s, 18, 30, 29, 21, 28, 19-H_3), 1.55, 1.57 (3H each, all s, 26, 27-H_3, reversible), 2.01 (3H, s, –OC(O)CH_3), 3.57 (1H, br s, 10-H), 4.35 (1H, m, 16-H), 6.50 (1H, d, *J* = 15.6 Hz, 23-H), 7.05 (1H, d, *J* = 15.6 Hz, 24-H). ^{13}C NMR (68 MHz, $CDCl_3$) δ_C: 18.2, 19.9, 20.1, 20.2, 21.9, 23.5, 24.0, 24.3, 25.1, 25.6, 25.9, 26.0, 26.3, 27.2, 27.9, 28.0, 28.8, 29.0, 33.4, 35.7, 35.8, 36.3, 39.0, 39.1, 40.5, 40.9, 41.4, 45.5, 48.0, 48.4, 48.9, 49.3, 50.5, 55.6, 58.2, 60.2, 61.7, 71.2, 78.2, 79.3, 120.4, 121.6, 132.1, 135.8, 143.1, 151.9, 163.8, 170.3, 171.7, 173.2, 173.2, 195.4, 202.5, 213.1. Positive-ion FAB-MS: *m*/*z* 1009 $[M+H]^+$, 1031 $[M+Na]^+$.
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