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Gliotoxin from the marine fungus *Aspergillus fumigatus* induces apoptosis in HT1080 fibrosarcoma cells by downregulating NF- κ B

Young-Sang Kim and Sun Joo Park*

Abstract

Gliotoxin has been recognized as an immunosuppressive agent for a long time. Recently, it was reported to have antitumor properties. However, the mechanisms by which it inhibits tumors remain unclear. Here, we showed that gliotoxin isolated from the marine fungus *Aspergillus fumigatus* inhibited proliferation and induced apoptosis in HT1080 human fibrosarcoma cells. Gliotoxin repressed phosphorylation-dependent degradation of I κ B- α , an antagonist of nuclear factor kappa B (NF- κ B), which is a known tumor-promoting factor. This coincided with a decrease in nuclear import of NF- κ B, suggesting its signaling activity was impaired. Moreover, gliotoxin increased intracellular reactive oxygen species (ROS). Since ROS have been known to inhibit NF- κ B, this may also contribute to gliotoxin's antitumorigenic effects. These results suggest that gliotoxin suppressed the activation of NF- κ B by inhibiting phosphorylation and degradation of I κ B- α and by increasing ROS, which resulted in apoptosis of HT1080 cells. Cumulatively, gliotoxin is a promising candidate antagonist of NF- κ B, and it should be investigated for its possible use as a selective inhibitor of human fibrosarcoma cells.

Keywords: Gliotoxin, Marine fungus, *Aspergillus fumigatus*, Apoptosis, HT1080 cells

Background

Fungal secondary metabolites are chemically diverse, and some display pharmaceutical potential as inhibitors of inflammation, cytotoxicity, and cancer (Shao et al. 2011; Wei et al. 2010). Gliotoxin, a member of the epipolythiodioxopiperazine family of secondary metabolites, is characterized by a disulfide bond across a piperazine ring. It has been isolated from several species of fungi, including the human pathogen *Aspergillus* (Waring et al. 1995). Gliotoxin is known to possess immunosuppressive properties, and it causes apoptosis in immune cells including neutrophils, eosinophils, macrophages, and thymocytes (Sutton et al. 1994; Pahl et al. 1996; Lopez-Franco et al. 2002). Gliotoxin's immunosuppressive properties result from its inhibition of nuclear factor kappa B (NF- κ B) because it represses proteasome-mediated degradation of the NF- κ B antagonist, I κ B- α

(Kroll et al. 1999; Lopez-Franco et al. 2002). Gliotoxin also enters the redox cycle and transitions between reduced and oxidized forms after uptake into cells. Reactive oxygen species (ROS) are produced as a by-product, and this induces DNA damage and apoptosis in immune cells (Harms et al. 2015). ROS have also been reported to inhibit NF- κ B through direct oxidation (Toledano and Leonard 1991; Nowak et al. 2008). In this regard, the role of gliotoxin in regulating NF- κ B and ROS has focused on immunological therapeutic applications. However, recent publications suggest that gliotoxin has antitumor properties (Kweon et al. 2003; Bhatnagar and Kim 2015), although the mechanistic details remain unclear. In the present study, we investigated the anti-proliferative effects of gliotoxin on HT1080 human fibrosarcoma cells. We found that gliotoxin from the marine fungus *Aspergillus fumigatus* regulates proliferation of HT1080 cells by inducing apoptosis. This cytotoxicity was related to the inactivation of NF- κ B and an increase in intracellular ROS.

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Methods

Materials and chemicals

Gliotoxin from the marine fungus *Aspergillus fumigatus* was obtained as described by Bhatnagar and Kim (2015). Briefly, *A. fumigatus* was isolated from the surface of marine green algae, which were collected in Cheongsapo, Busan, Republic of Korea. The fungal strain was cultured in YPGA media containing 0.1 % yeast extract, 2 % peptone, 1 % glucose, 2 % agar, 60 % seawater, and 40 % distilled water. The fermentation broth was extracted (2.3 g) with ethyl acetate (EtOAc, 1:1.5 v/v; broth-EtOAc, 1:1 v/v). The extract was fractionated by silica gel chromatography (*n*-hexane-EtOAc, 100 %:0 %; CH₂Cl₂-methyl alcohol (MeOH), 1:1), ODS column chromatography (H₂O-MeOH, 100 %:100 %), and Sephadex LH-20 column chromatography (H₂O-MeOH, 100 %:100 %). High-performance liquid chromatography (HPLC) using a YMC ODS-A column (250 mm × 10 mm I.D., S-5 μm, 12 nm, MeOH) yielded purified gliotoxin (5.2 mg). The structure and molecular formula of gliotoxin was ascertained using ¹H and ¹³C nuclear magnetic resonance (NMR), as well as low-resolution electron impact mass spectrometry (LREI-MS) data. ¹H NMR spectrum (DMSO-d₆, 400 MHz): 3.44 (1H, dd, *J* = 4.8, H-3a), 4.28 (1H, dd, *J* = 9.9, H-3a), 4.39 (1H, dd, *J* = 6.8, H-5), 4.842 (1H, m, H-6), 5.78 (1H, d, *J* = 9.9, H-7), 5.95 (1H, m, H-8), 6.00 (1H, m, H-9), 2.96, 3.73 (1H, d, *J* = 18.1, H-10), 3.20 (3H, s, H-11). ¹³C NMR spectrum (DMSO-d₆, 100 MHz): 166.0 (C-1), 77.2 (C-3), 60.5 (C-3), 165.2 (C-4), 69.8 (C-5), 75.6 (C-6), 129.9 (C-7), 123.4 (C-8), 120.2 (C-9), 130.7 (C-19a), 36.6 (C-10), 73.1 (C-10a), 27.5 (C-11). LREI-MS *m/z*: 349 [M]⁺ (C₂₁H₂₃N₃O₂). LREI-MS *m/z*: 326.38 [M]⁺ (C₁₃H₁₄N₂O₄S₂).

Human fibrosarcoma cells (HT1080) were obtained from the Korean Cell Line Bank (KCLB, Seoul, Korea). Cells were cultured in DMEM supplemented with penicillin/streptomycin and fetal bovine serum (FBS) (Gibco BRL, Life Technology, NY, USA). Cytotoxicity assays were performed with 3-(4,5-dimethyl-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) reagent (Sigma Chemical Co., St. Louis, MO, USA). All antibodies used for western blot analysis were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Other chemicals and reagents were of commercially available analytical grade.

Cell culture and cell viability assay

HT1080 cells were cultured on DMEM (Gibco, USA) containing 10 % FBS and incubated at 37 °C under a humidified atmosphere of 5 % CO₂. Cytotoxicity of gliotoxin on HT1080 cells was analyzed using an MTT assay. To estimate the cytotoxicity of gliotoxin, cells were seeded in a 96-well plate at a concentration of 4 × 10⁴ cells/ml and incubated for 24 h. The cells were then

exposed to different concentrations (0, 25, 50, or 100 μM) of gliotoxin at 37 °C under a humidified atmosphere of 5 % CO₂. Subsequently, 100 μl MTT solution (1 mg/ml) was added to each well, and cells were incubated for another 3 h. The formazan salt that formed was dissolved in 100 μl DMSO. The absorbance was measured at 550 nm (GENiosr Microplate Reader, Tecan Austria GmbH, Austria).

Annexin V/propidium iodide (PI) assay

Apoptotic cells were stained using the fluorescence isothiocyanate (FITC) Annexin V apoptosis detection kit (BD Biosciences) according to the manufacturer's instructions. Briefly, HT1080 cells were cultured in 6-well plates at a density of 5 × 10⁴ cells/ml. The cells were then treated with 100 μM gliotoxin for 24 h. After incubation, trypsinized, floating, and adherent cells were pooled and centrifuged. Harvested cells were washed with PBS twice, mixed in 1× binding buffer, and incubated with Annexin V/PI double-staining solution at room temperature (RT) for 20 min. The stained cells were analyzed by flow cytometry (FACSCalibur, BD Science, Heidelberg, Germany), and the percentage of apoptotic cells was calculated using Cell Quest software.

Extraction of cellular proteins

After the indicated incubation times with or without 100 μM gliotoxin, HT1080 cells were lysed. Nuclear and cytoplasmic extracts were prepared according to Duvoix et al. (2004). Briefly, cell pellets were suspended in ice-cold hypotonic lysis buffer containing protease inhibitors. We added 10 % Igepal (Sigma-Aldrich) to the cell suspension and vortexed for 10 s to lyse the cells. The lysate was then centrifuged at 18,000 rpm for 1 min. The cytoplasmic supernatant was stored, and cell pellets were washed with hypotonic lysis buffer. Nuclear extraction buffer was added to each pellet, and the cell suspension was gently mixed on an orbital shaker for 15 min at 4 °C. This was followed by centrifugation at 10,500 rpm for 7 min at 4 °C. Finally, the nuclear supernatant was transferred to a prechilled microcentrifuge tube and stored at -80 °C.

Western blot analysis

Western blotting was performed according to the standard procedures. Briefly, cells were cultured at a density of 5 × 10⁴ cells/ml in six-well plates with serum-free media. After incubation for 24 h, cells were treated with gliotoxin for another 24 h. Cells were lysed in RIPA (150 mM NaCl, 1.0 % IGEPAL® 0.5 % sodium deoxycholate, 0.1 % sodium dodecylsulfate, 50 mM Tris, pH 8.0) buffer at 4 °C for 30 min. Proteins from nuclear and cytoplasmic extracts were obtained, and 100 μg/ml of

protein was separated using 10 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis with a 5 % stacking gel. Separated proteins were transferred onto a nitrocellulose membrane (Amersham Pharmacia Biotech, England, UK). The membrane was blocked for 1.5 h at RT using Tris-buffered saline and 0.1 % Tween-20 (TBS-T) with 5 % skim milk. After washing the membrane with TBS-T twice, the blots were incubated for 1 h with the indicated antibodies at 25 °C. The respective proteins were detected with a chemiluminescent ECL assay kit (Amersham Pharmacia, England, UK) according to the manufacturer's instructions.

Measurement of ROS

HT1080 cells (2×10^5 cells) were incubated with or without 100 μM gliotoxin for 24 h. Cellular ROS levels were determined by staining with dihydroethidium (DHE) (Sigma-Aldrich) and by using the Muse Oxidative Stress Kit (Millipore) according to the manufacturer's instructions.

Statistical analysis

Data were analyzed using the InStat statistics program (GraphPad Software, Inc., San Diego, CA, USA). Statistical comparisons were performed using one-way analysis of variance (ANOVA) with the Bonferroni test for multiple comparisons.

Results

To examine the antiproliferative effects of gliotoxin on human HT1080 fibrosarcoma cells, we treated cultures with various concentrations of gliotoxin (0–100 μM) for 24 h. The antiproliferative effects were assessed by a colorimetric MTT assay. Gliotoxin treatment decreased the number of viable cells in a dose-dependent manner (Fig. 1). This suggested that gliotoxin decreased proliferation of HT1080 cells, and it may have generally cytotoxic effects.

To determine whether the antiproliferative effect of gliotoxin was associated with apoptotic cell death, HT1080 cells were treated with 100 μM gliotoxin for 24 h. Cells were then double stained with Annexin V and propidium iodide (PI), and flow cytometric analyses were performed (Fig. 2). The percentage of Annexin V-positive and PI-positive cells presenting in late-apoptosis phase increased from 33.7 to 68.7 % after treatment with gliotoxin. This clearly indicates that gliotoxin induced apoptosis in HT1080 cells.

NF- κB induces expression of several genes that promote malignant properties associated with tumor growth and inhibit apoptosis (Panwalkar et al. 2004). Constitutive activation of NF- κB has been shown in several types of cancer (Sasaki et al. 2001; Weichert et al. 2007; Sakamoto et al. 2009). In unstimulated cells, NF- κB is typically sequestered in the cytoplasm where

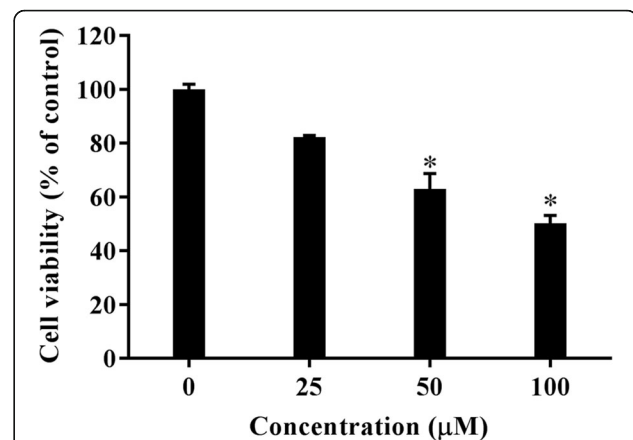


Fig. 1 Gliotoxin decreases proliferation of HT1080 human fibrosarcoma cells. HT1080 cells were treated with various concentrations (0 to 100 μM) of gliotoxin for 24 h. Cell viability was determined by MTT assay. Each value is expressed as the mean \pm standard deviation (SD) of three independent experiments. * $P < 0.05$ compared to the control group (0 μM)

it complexes with inhibitors such as I κB - α . When cells experience the proper stimulus, cytoplasmic NF- κB becomes activated primarily through phosphorylation and degradation of I κB - α . NF- κB then translocates from the cytosol to the nucleus and induces expression of target genes (Siebenlist et al. 1994; Baeuerle and Baltimore 1996).

To examine the effects of gliotoxin on NF- κB signaling, we analyzed the expression and phosphorylation state of I κB - α . We also investigated the expression of NF- κB and the nuclear translocation dynamics of the p65 subunit after gliotoxin treatment (Fig. 3). HT1080 cells were cultured with gliotoxin (0–100 μM) for 24 h, and the cytosol and nuclear lysates were each subjected to western blot analysis. Treatment with gliotoxin inhibited NF- κB activation. High levels of NF- κB -p65 were found in nuclear fraction of control HT1080 cells without gliotoxin treatment. However, exposure to gliotoxin decreased the amount of nuclear-localized NF- κB -p65. Gliotoxin treatment also reduced phosphorylation of I κB - α and increased overall expression of I κB - α . Together, these results suggest that gliotoxin inhibited the nuclear import and activation of NF- κB by suppressing the phosphorylation and subsequent degradation of its inhibitor, I κB - α .

ROS are produced by a variety of cellular processes. This oxidative stress response frequently triggers apoptotic cell death (Russell and cotter 2015). One reason for this is the inhibition of NF- κB activity by ROS, which ultimately enhances apoptosis. Specifically, ROS inhibit DNA binding of NF- κB through direct oxidation of NF- κB (Toledano and Leonard 1991; Nowak et al. 2008) and through the modification of its inhibitor,

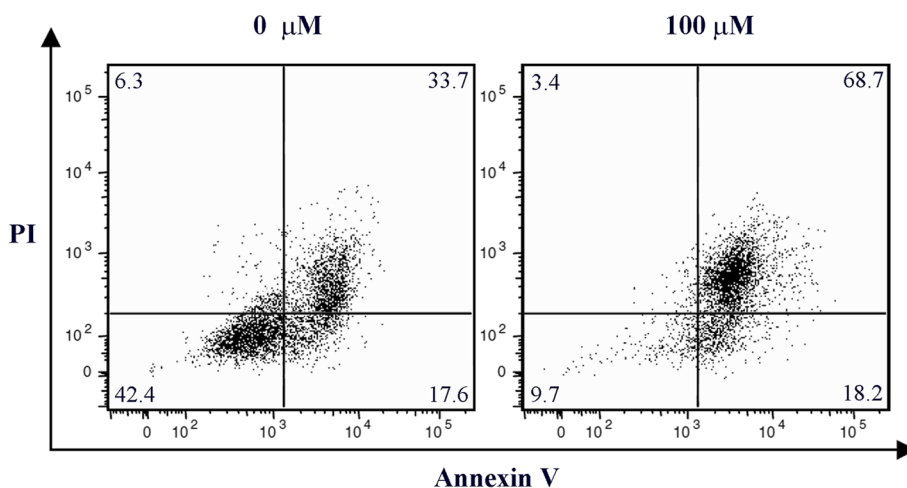


Fig. 2 Gliotoxin induces apoptosis of HT1080 cells. HT1080 cells were treated with 100 μM gliotoxin for 24 h and analyzed by Annexin V/PI staining. Gliotoxin increased the cell population occupying the Annexin V/PI double-positive quadrant (UR upper right), which represents late-apoptotic cells

IκB-α. Therefore, we examined whether gliotoxin influenced ROS levels in HT1080 cells. After treating cells with 100 μM gliotoxin, ROS levels increased from 32.2 to 62.7 % compared to untreated controls (Fig. 4). Taken together, these results suggest that gliotoxin triggered apoptotic cell death in HT1080 cells via generation of ROS and inactivation of NF-κB signaling.

Discussion

Apoptosis is a process of programmed cell death that occurs in multicellular organisms. The development and progression of cancer frequently involves the suppression of apoptosis. Therefore, almost all cytotoxic anticancer therapies currently in clinical use attempt to target cancer cells by inducing apoptosis. The NF-κB pathway is involved in tumorigenesis, tumor cell proliferation, and

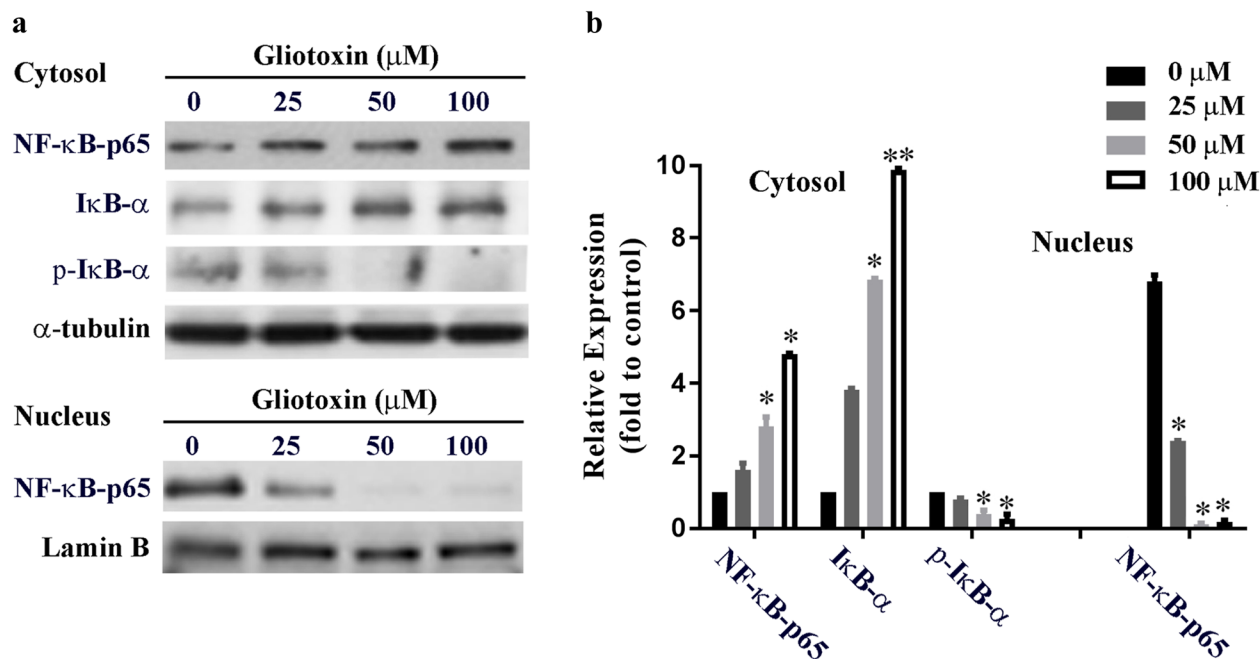
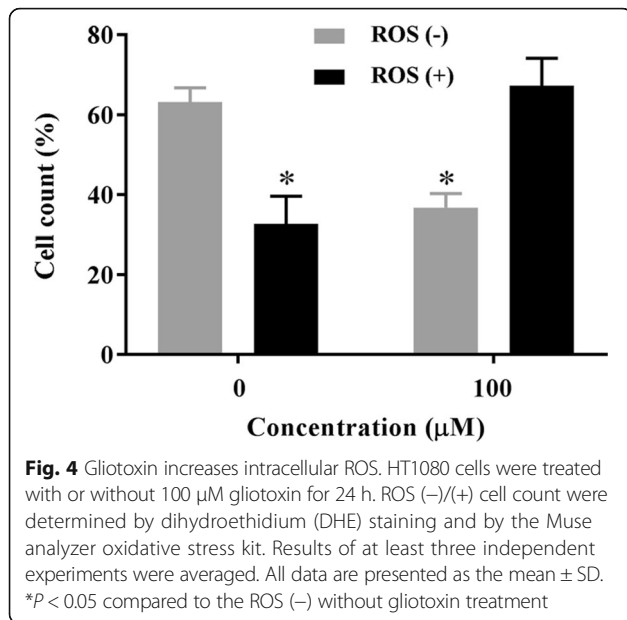


Fig. 3 Gliotoxin inhibits activation of NF-κB by suppressing the phosphorylation and degradation of IκB-α. HT1080 cells were treated with various concentrations (0 to 100 μM) of gliotoxin for 24 h, and the cytosol and nuclear lysates were subjected to immunostaining for the p65 subunit of NF-κB, phosphorylated IκB-α, total IκB-α, α-tubulin, and lamin B (a). The immunoblot signals were quantified. Each value was expressed as the mean ± SD of a triplicate experiment. *P < 0.05 compared to the control without gliotoxin treatment (b)



apoptosis, as well as cellular immunity and inflammation. For example, constitutive activation of NF- κ B has been reported in several types of cancers including gastric cancer, pancreatic cancer, and colorectal carcinoma (Sasaki et al. 2001; Weichert et al. 2007; Sakamoto et al. 2009). NF- κ B helps activate downstream proteins that promote tumor growth and inhibit apoptosis in tumor cells. Given the antiapoptotic effects of NF- κ B, inhibitors of NF- κ B are promising antitumor drugs. As a result, much effort has focused on developing inhibitors that target NF- κ B (Yemelyanov et al. 2006; Yang et al. 2006). Recently, ROS have been also reported to repress NF- κ B signaling in many ways. Direct oxidation of NF- κ B by ROS inhibits its DNA-binding ability (Toledano and Leonard 1991). ROS modification of I κ B- α leads to inhibition of NF- κ B signaling (Nowak et al. 2008). In addition, ROS stabilize I κ B- α by inhibiting the proteasome, resulting in downregulation of NF- κ B signaling (Wu et al. 2009). Therefore, it seems reasonable that ROS function as physiological signaling modulators of NF- κ B signaling cascades in cancers. Our results support this hypothesis. We showed that gliotoxin, a natural compound isolated from the marine fungus *A. fumigatus*, inhibited proliferation and induced apoptosis in human HT1080 fibrosarcoma cells by increasing intracellular ROS levels and repressing activation and nuclear translocation of NF- κ B. Therefore, we suggest that gliotoxin may constitute a promising new antitumor drug for the targeted inhibition of NF- κ B signaling.

Conclusions

We demonstrated that gliotoxin, a mycotoxin produced by the marine fungus *A. fumigatus*, induced apoptosis in

HT1080 human fibrosarcoma cells. It achieved this, at least in part, through an increase in levels of intracellular ROS and repression of NF- κ B signaling. Cumulatively, our results provide a new avenue of research into the design and development of NF- κ B antagonists for the treatment of cancer.

Abbreviations

DHE: Dihydroethidium; EtOAc: Ethyl acetate; FBS: Fetal bovine serum; HPLC: High-performance liquid chromatography; KCLB: Korean Cell Line Bank; LREI-MS: Low-resolution electron impact mass spectrometry; MeOH: Methyl alcohol; MTT: 3-(4,5-Dimethyl-2-yl)-2,5-diphenyltetrazolium bromide; NF- κ B: Nuclear factor kappa B; NMR: Nuclear magnetic resonance; PI: Propidium iodide; ROS: Reactive oxygen species; RT: Room temperature

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Availability of data and materials

All datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YSK designed and carried out the experiments. SJP interpreted the results and wrote the manuscript. Both authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Not applicable.

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