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Active compounds from *Saussurea lappa* Clarks that suppress hepatitis B virus surface antigen gene expression in human hepatoma cells

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Abstract

We have examined the antiviral activity of the crude extract prepared from the root of *Saussurea lappa* Clarks, a Chinese medicinal herb which is widely used for many illneses including cancer. Two active components, costunolide and dehydrocostus lactone, were identified which show strong suppressive effect on the expression of the hepatitis B surface antigen (HBsAg) in human hepatoma Hep3B cells, but have little effect on the viability of the cells. Both costunolide and dehydrocostus lactone suppress the HBsAg production by Hep3B cells in a dose-dependent manner with IC_{50}s of 1.0 and 2.0 μM, respectively. Northern blotting analysis shows that the suppression of HBsAg gene expression by both costunolide and dehydrocostus lactone were mainly at the mRNA level. Furthermore, the suppressive effect of costunolide and dehydrocostus lactone on HBsAg and hepatitis B e antigen (HBeAg), a marker for hepatitis B viral genome replication in human liver cells, was also observed in another human hepatoma cell line $HepA_2$ which was derived from $HepG_2$ cells by transfecting a tandemly repeat hepatitis B virus (HBV) DNA. Similarly, the mRNA of HBsAg in $HepA_2$ cells was also suppressed by these two compounds. Our findings suggest that costunolide and dehydrocostus lactone may have potential to develop as specific anti-HBV drugs in the future.

Keywords: Saussurea lappa Clarks; Sesquiterpene lactone; Antiviral agent; Gene regulation

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1. Introduction

Infection by hepatitis B virus (HBV) frequently results in acute and chronic hepatitis and is also associated with a high risk of developing primary hepatocellular carcinomas in the human (Brechot et al., 1980; Beasley et al., 1981; Shafritz et al., 1981; Lo et al., 1982). Although immunization against HBV has been shown to be effective in preventing chronic infections (Lo et al., 1988), effective drugs to eradicate HBV in chronic carriers are still not available.

Recently, oral preparation of *Phyllanthus amarus*, an Indian medicinal herb, was shown by Thyagarajan and colleagues (Thyagarajan et al., 1976) in a clinical trials to enhance the seroconversion rate of HBsAg in chronic carriers of HBV (Thyagarajan et al., 1976; Venkateswaran et al., 1987; Blumber et al., 1989; Niu et al., 1990). We have shown that the *P. amarus* extract suppressed transcription of the surface antigen (HBsAg) gene of HBV in $HepA_2$ cells (Yeh et al., 1993), a cell line derived from human hepatoma $HepG_2$ cells by transfecting a tandemly repeated HBV DNA (Aden et al., 1979). These cell lines, therefore, can be developed as a rapid assay system for screening biological active components from natural resources to regulate HBV viral gene expression.

In this study, we report that two active substances were identified from the Chinese herbal medicine, *Saussurea lappa* Clarks which suppressed HBsAg gene expression in both Hep3B and $HepA_2$ cells. The structures of these two compounds were identified as costunolide and dehydrocostus lactone, respectively.

2. Materials and methods

2.1. Materials

Hepatitis B surface antigen (HBsAg) and e antigen (HBeAg) enzyme immunoassay (EIA) kits were purchased from Ever New Co. (Taipei, Taiwan). Fetal calf serum was obtained from Hyclone (Logan, UT, USA). Dulbecco's modified Eagle's medium (DMEM) was obtained from Gibco/BRL (Gaithersbung, MD, USA). [α-^{32}P]dCTP (3000 Ci/mmol) was obtained from Amersham (Little Chalfont, Bucks, UK). Sea Kem LE-agarose was purchased from FMC Bioproducts (Rockland, MA, USA).

2.2. Cell culture

Stock cultures of human hepatoma cells Hep3B and $HepA_2$ were maintained in DMEM medium supplemented with 10% fetal calf serum and antibiotics (100 IU/ml each of penicillin and streptomycin) in a humidified atmosphere containing 5% CO_2 and 95% air at 37°C. The cultures were passaged by trypsinization every 4 days. For bioassays, cells were plated either in 24-well plates at a density of 1.0×10^5 cells/well or in 100-mm culture dishes at a density of 1.5×10^6 cells/dish in DMEM medium containing 10% fetal calf serum.

2.3. Preparation of tested compound

The root of *S. lappa* Clarks was sliced and extracted with methanol and subsequently partitioned in *n*-hexane/water (1 : 1, v/v). The *n*-hexane soluble material was fractionated by *n*-hexane and ethyl acetate gradient of sequential column chromatography in silica-gel. The active component was eluted in *n*-hexane/EtOAc (95 : 5, v/v) and was further purified by reverse-phase high-performance liquid chromatography to homogeneity. For bioassay, the compound was first dissolved in ethanol, filtered through a 0.25-μm fluoropore filter (Millipore), and added to cell cultures.

2.4. Determination of HBsAg and HBeAg

Human hepatoma cells, Hep3B or $HepA_2$, were seeded in 24-well plates at a density of 1.0×10^5 cells/well in DMEM medium containing 10% fetal calf serum. After 24 h of incubation, cells were washed three times with phosphate-buffered saline (pH 7.0) and treated with various concentrations of drugs in serum-free DMEM for 48 h. The HBsAg and HBeAg in culture medium were measured by enzyme immunoassay (EIA) kits. The viability of cells was determined by trypan blue exclusion and counted in a hemocytometer.

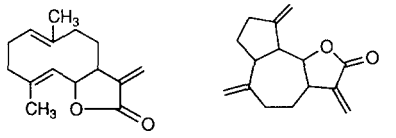
2.5. RNA extraction and Northern blotting

Total cellular RNA was isolated by centrifugation through cesium chloride according to the method of Glisin et al. (1974). The RNA (20 μg) was denatured in 6.5% formaldehyde and fractionated by electrophoresis in a 1% agarose gel. The RNA was then transferred to nitrocellulose filter (Thomas, 1983) by capillary blotting and immobilized by heating at 80°C for 2 h. The membrane filters were prehybridized for 6 h at 42°C in a solution containing 35% formamide, 5 × Denhardt's reagent (1 × = 0.02% ficoll, 0.02% BSA, 0.02% polyvinyl-pyrrolidone), 5 × SSPE (1 × = 0.15 M NaCl, 0.01 M NaH_2PO_4, 1 mM EDTA (pH 7.4), 1% SDS, and 500 μg/ml denatured salmon sperm DNA. A full-length HBV viral DNA was labeled with [α-^{32}P]dCTP by random primer. Denatured ^{32}P-labeled DNA probe (10^6 cpm/μg) was added directly to the prehybridization buffer, and hybridization was carried out for 36 h at 42°C. The membrane filters were washed twice for 15 min in 2 × SSC (1 × SSC, 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.0), 0.2% SDS at 42°C, and once for 15 min in 0.1 × SSC, 0.1% SDS at 65°C. Autoradiography was performed at −70°C with an intensifying screen (Quanta IV, Du Pont) for 12 h on Kodak SAFET X-ray film.

3. Results

3.1. Structure of costunolide and dehydrocostus lactone

In the course of our continuing search for natural plant products as anti-HBV agents, the MeOH extract of the root of *S. lappa* Clarks was found to show significant



Costunolide Dehydrocostus lactone

Fig. 1. Chemical structures of costunolide and dehydrocostus lactone.

anti-HBsAg activity. Subsequent bioactivity-directed fractionation has resulted in the isolation of compounds A and B as the active agents. The chemical structures of the two pure active compounds A and B were determined by the combination of mass spectrometry, ^{1}H-NMR, ^{13}C-NMR, infrared (IR), and ultraviolet (UV) spectra. The UV spectra of compounds A and B in CH_2Cl_2 showed almost identical absorption bands at 228 nm, indicating that they both possessed a α,β-unsaturated ketone skeleton. The infrared spectrum showed a γ-lactone of 5-member ring at 1760 cm^{-1}, a terminal alkene group around 910 cm^{-1} for both of compounds A and B, and a *trans*-alkene group at 965 cm^{-1} for compound A. On mass spectroscopy, the molecular ion peak for compounds A and B were detected at m/z 232 ($C_{15}H_{20}O_2$), 230 ($C_{15}H_{18}O_2$) (EIMS, 12 eV), respectively. Further spectroscopic data (^{1}H-, ^{13}C-NMR, DEPT, HH-COSY, CH-correlation) confirmed the chemical structures of compounds A and B to be costunolide and dehydrocostus lactone, respectively (Rucker et al., 1991; Zdero et al., 1991a; Ali et al., 1992; Lonergan et al., 1992) (Fig. 1.)

3.2. Suppression of HBsAg production in Hep3B cells

The Hep3B cells were plated into 24-well plates and allowed to attach overnight. The medium was changed to DMEM without serum, and various concentrations of costunolide or dehydrocostus lactone were added. After 48 h of incubation, HBsAg produced in the culture medium was determined. The results showed that both costunolide and dehydrocostus lactone severely suppressed HBsAg production by the Hep3B cells with IC_{50}s of about 1.0 and 2.0 μM, respectively. These two compounds by themselves did not interfere with the enzyme immunoassay of HBsAg determination (data not shown). The HBsAg suppression activities of costunolide and dehydrocostus lactone were not due to any cytotoxicity of these two compounds, since the treated cells were still viable and continued to proliferate slowly during the 48-h incubation period (Fig. 2).

3.3. The mRNA of HBsAg in drug-treated Hep3B cells

To test whether costunolide and dehydrocostus lactone reduced the mRNA of the HBsAg gene in the Hep3B cells, we examined the change of HBsAg mRNA during the 48 h of drug treatment by Northern blot hybridization using HBV DNA as the probe (Fig. 3). One major mRNA species, 4.0 kb in length, was detected by hybridization. The dramatic decrease of HBsAg mRNA in the Hep3B cells during the drug treatment suggested that the suppression of HBsAg gene expression in Hep3B cells by both costunolide and dehydrocostus lactone was mainly at the mRNA level (Fig. 3).

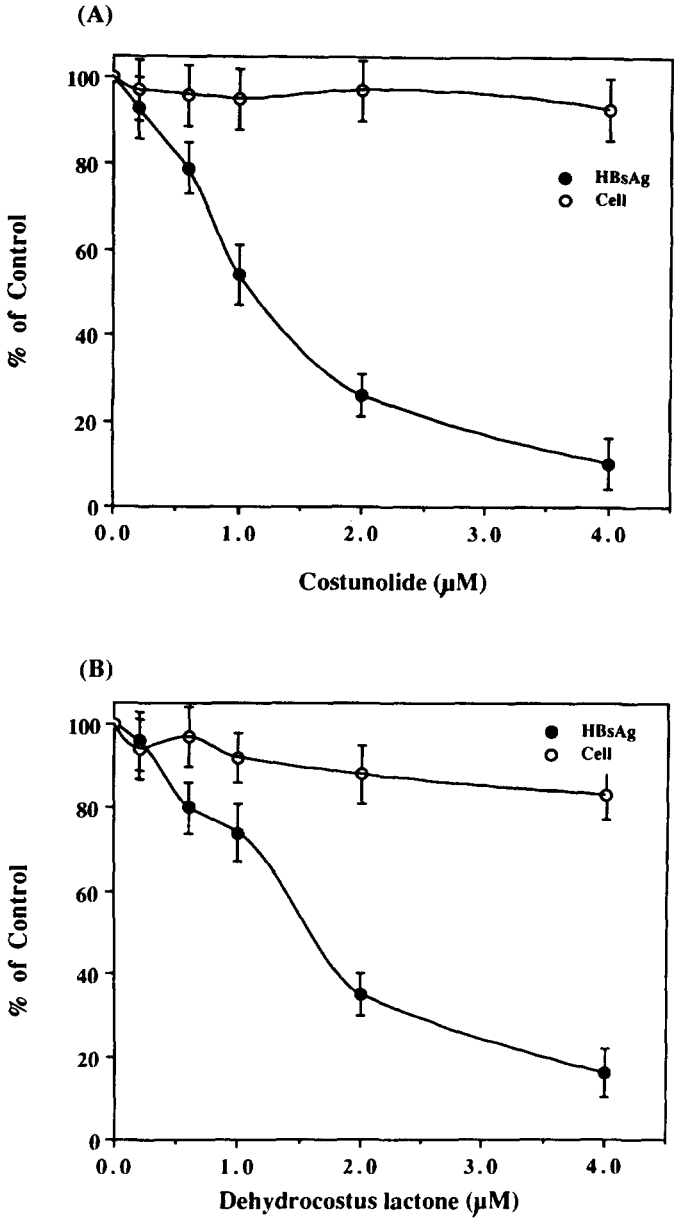


Fig. 2. Effect of costunolide and dehydrocostus lactone on HBsAg production and cell proliferation in Hep3B human hepatoma cells. Hep3B cells were seeded on 24-well plates at a density of 1.0×10^5 cells/cm^2 in DMEM with 10% fetal calf serum and allowed to attach overnight. The cells was then washed three times with phosphate-buffered saline (pH 7.0) and treated with various concentrations of costunolide (A) and dehydrocostus lactone (B) in serum-free DMEM medium for another 48 h. The amount of HBsAg in the culture medium was determined by enzyme immunoassay. Viable cells in each well were determined by trypan blue exclusion and counted in a hemocytometer. Data are expressed as mean $\pm$ S.D. ($n = 3$).

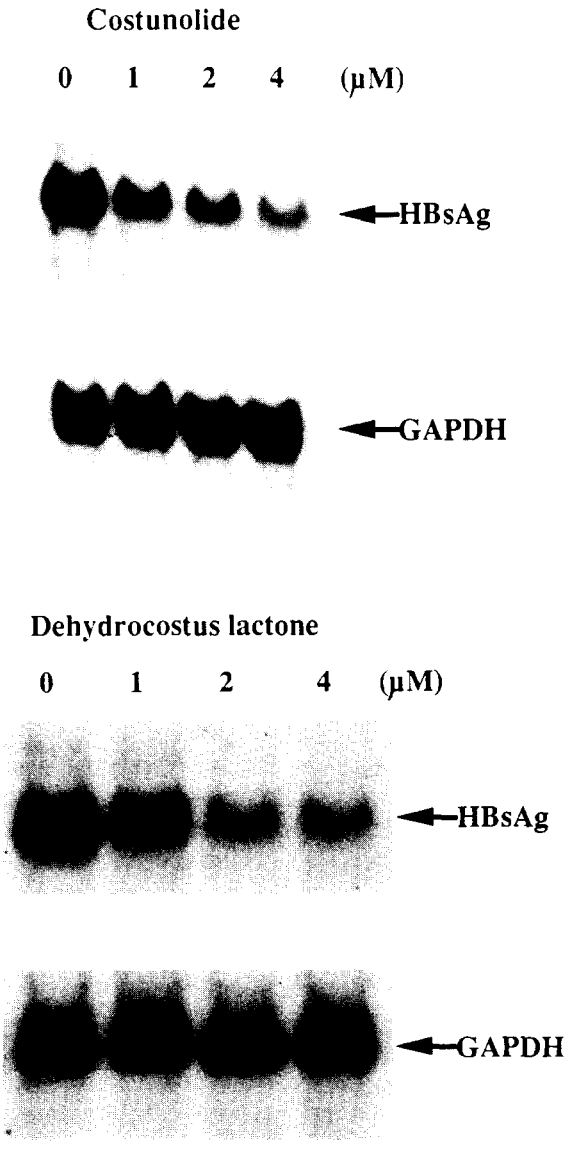


Fig. 3. Dose-dependent effect of costunolide and dehydrocostus lactone on the steady-state mRNA level of HBsAg in Hep3B cells. Hep3B cells were seeded on 100 mm culture dishes and treated with 0, 1, 2, and 4 μM of costunolide or dehydrocostus lactone in serum-free DMEM medium for 48 h. Total RNA was extracted and analyzed by Northern hybridization with HBV viral DNA probe as described in Materials and methods. The mRNA of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as an internal marker.

3.4. Effect of HBsAg and HBeAg production on HepA_2 cells

To investigate whether the suppression of HBsAg gene expression by costunolide and dehydrocostus lactone could be observed in other human hepatoma cells. We tested

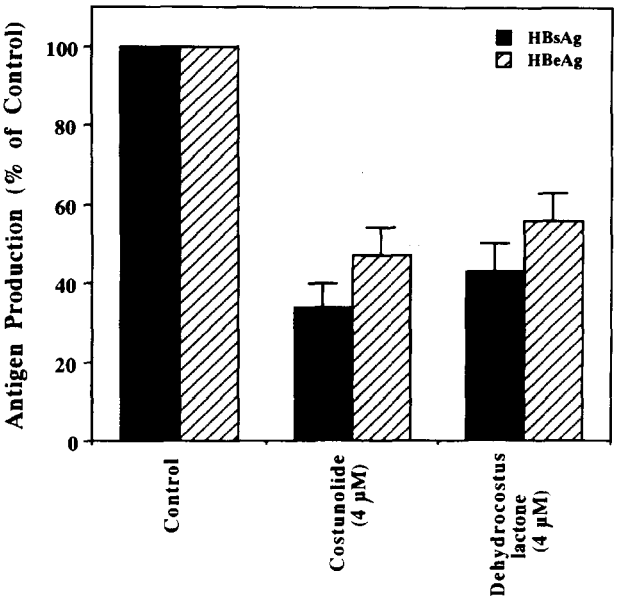


Fig. 4. The effect of costunolide and dehydrocostus lactone on HBsAg and HBeAg production in $HepA_2$ cells. $HepA_2$ cells were treated with costunolide (4 μM) or dehydrocostus lactone (4 μM) in serum-free DMEM medium for 48 h as described in the legend to Fig. 2. Viable cells in each well were determined by trypan blue exclusion and counted in a hemocytometer. The amount of HBsAg and HBeAg in the culture medium was determined by enzyme immunoassay. Control cells produced 15 ng of HBsAg/10^6 cells/48 h and 1.2 ng of HBeAg/10^6 cells/48 h. Data are expressed as mean $\pm$ S.D. ($n = 3$).

another human hepatoma cell line, $HepA_2$, which was derived from $HepG_2$ cells by transfecting a tandemly repeated full-length HBV DNA (Aden et al., 1979). The $HepA_2$ cells continually synthesize and secrete not only HBsAg, but also HBeAg, a marker for viral replication. When the $HepA_2$ cells were treated with either costunolide or dehydrocostus lactone for 48 h in the serum-free medium, the production of both HBsAg and HBeAg by the $HepA_2$ cells was suppressed (Fig. 4). The suppression of HBsAg gene expression in the $HepA_2$ cells by costunolide and dehydrocostus lactone was also shown to be at least partly at the mRNA level (Fig. 5). Moreover, neither costunolide nor dehydrocostus lactone had any cytotoxic effect on the $HepA_2$ cells (data not shown).

4. Discussion

In this study, we identified two active substances which showed strong suppressive effect on HBsAg gene expression from the Chinese herbal medicine, *S. lappa* Clarks. The spectral data of these two pure compounds were assigned for germacranolide and guaianolide sesquiterpene lactone as costunolide and dehydrocostus lactone, respectively (Jakupovic et al., 1991; Zdero et al., 1991a,b).

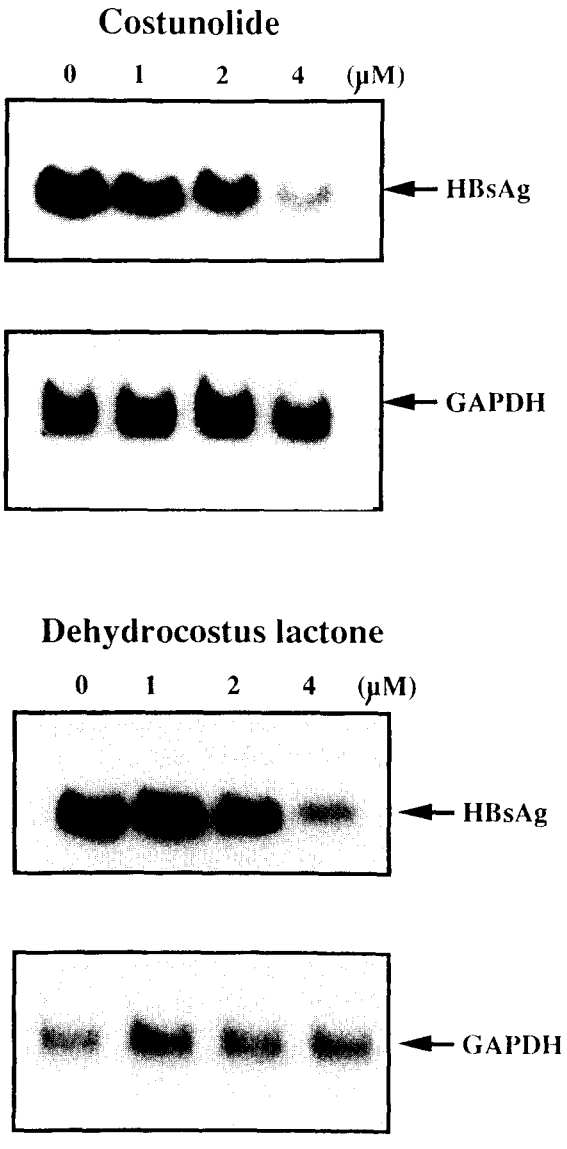


Fig. 5. Dose-dependent effect of costunolide and dehydrocostus lactone on HBsAg mRNA level in $HepA_2$ cells. $HepA_2$ cells were seeded on 100 mm culture dishes and treated with 0, 1, 2, and 4 μM of costunolide or dehydrocostus lactone in serum-free DMEM medium for 48 h. Total RNA was extracted and analyzed by Northern blot hybridization as described in Materials and methods. The mRNA of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as an internal control. The experiment presented here was performed three times with similar results.

γ-Lactone-bearing sesquiterpene lactones have previously been reported to have antimicrobial and antifeedent activity (Lonergan et al., 1992). However, our results show, for the first time, that the costunolide and dehydrocostus lactone can suppress

HBsAg production in human hepatoma cells. It has been argued that the binding of surface antigen or chelating properties of the active agent(s) in *P. amarus* would be an important mechanism in the clearance of the HBsAg in the serum of infected individuals (Thyagarajan et al., 1988). However, the costunolide and dehydrocostus lactone could not interfere the ELISA assay for HBsAg had ruled out such possibility.

Cultured human hepatoma Hep3B cells contain one to two copies of HBV genomes in their chromosomes (Twist et al., 1981) and actively secrete HBsAg into the medium (Aden et al., 1979). We have previously shown that the Hep3B cells express one major 4.0-kb mRNA which contains only one entire HBsAg coding sequence (Su et al., 1986; Chou et al., 1989). The 5′-end of this RNA has been mapped to the region close to the *Eco*RI site at nucleotide 3200, a region of the major surface antigen promoter and the 3′-end of viral sequence was mapped at DR region. The genomic analysis indicates that this RNA is directly transcribed from the major surface antigen promoter, but fused with some cellular sequence (data not shown). On the other hand, the $HepA_2$ cells express 2.1 kb mRNA which codes for HBsAg and also transcribed from the major surface antigen promoter in the transfected HBV DNA.

The mechanism of the actions of costunolide and dehydrocostus lactone towards HBsAg gene expression is still unknown. Since they not only lower the mRNA of HBsAg in two human hepatoma cell lines, but also have no effect on the transcript of a housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) suggest they might directly suppress promoter activity of the HBsAg gene. They could also block the enhancer activity of viral enhancer I (Chang et al., 1987) or enhancer II (Yuh et al., 1990) to suppress viral promoter activity. However, we cannot rule out the possibility that these compounds may specifically destablize viral mRNA in human liver cells.

Since costunolide and dehydrocostus lactone also suppress HBeAg production in $HepA_2$ cells, they might interfere with viral replication in the $HepA_2$ cells. However, the $HepA_2$ cell only produces very low levels of 3.5 kb pregenomic viral RNA. At present, we still have no clear data to show that costunolide and dehydrocostus lactone also strongly suppress viral replication in the $HepA_2$ cells. This hypothesis is currently under investigation in our laboratory.

Our results establish a rapid and convenient biological assay system for identifying more possible antiviral components from natural resources. The elucidation of the mechanism of anti-HBsAg activity of these natural products will greatly enhance our understanding on how the viral gene expressions are regulated and open many new clues to develop antiviral agents in the future.

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