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Pseudolaric acid B induces apoptosis *via* activation of c-Jun N-terminal kinase and caspase-3 in HeLa cells

Xianfeng Gong[1,2], Minwei Wang[2], Zhen Wu[1], Shin-ichi Tashiro[3], Satoshi Onodera[3] and Takashi Ikejima[1,4]

[1]China-Japan Research Institute of Medical Pharmaceutical Sciences
[2]Department of Pharmacology
Shenyang Pharmaceutical University
Shenyang, 110016, China
[3]Department of Clinical and Biomedical Sciences
Showa Pharmaceutical University
Tokyo 194-8543, Japan
[4]Coressponding author: Tel/Fax, 86-24-23844463;
E-mail, ikejimat@vip.sina.com

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Abbreviations: ERK, extracellular signal-regulated protein kinase; ICAD, inhibitor of caspase-activated DNase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PARP, poly (ADP-ribose) polymerase; z-DEVD-fmk, benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethylketone

Abstract

Pseudolaric acid B was isolated from *Pseudolarix kaempferi* Gordon (Pinaceae) and was evaluated for the anti-cancer effect in HeLa cells. We observed that pseudolaric acid B inhibited cell proliferation and induced apoptosis in a time- and dose-dependent manner. HeLa cells treated with pseudolaric acid B showed typical characteristics of apoptosis including the morphological changes and DNA fragmentation. JNK inhibitor, SP600125, markedly inhibited pseudolaric acid B-induced cell death. In addition, Bcl-2 expression was down-regulated while Bax protein level was up-regulated. Caspase-3 inhibitor, z-DEVD-fmk, partially blocked pseudolaric acid B-induced cell death, and the expression of two classical caspase substrates, PARP and ICAD, were both decreased in a time-dependent manner, indicative of downstream caspase activation.

Keywords: apoptosis; caspase; HeLa; MAPK; pseudolaric acid B

Introduction

The root and trunk bark of *Pseudolarix kaempferi* Gordon (Pinaceae), known as "Tu-Jin-Pi" in China, have been used in the treatment of dermatological fungi infections. Pseudolaric acid B (Figure 1), the diterpene acid isolated from *Pseudolarix kaempferi*, exerts potent antifungal, antimicrobial (Li *et al*., 1995), antifertility (Wang *et al*., 1982; Wang *et al*., 1988), and cytotoxic activities (Pan *et al*., 1990). Recently, pseudolaric acid B has been reported to induce apoptosis in Human promyelocytic leukemia, HL-60 (Zhang *et al*., 2002) and melanoma cell line, LiBr (Jiang *et al*., 2003), however, the precise mechanism of pseudolaric acid B-induced apoptosis is still unclear.

Apoptosis is an evolutionary conserved process that removes damaged or unwanted cells. It plays a critical role in vascular diseases (Han *et al*., 1995), immune related diseases (Lin, 2001), neurodegenerative disorders (Gibson, 2001), AIDS development (Oyaizu and Pahwa, 1995), as well as tumor therapy (Kerr *et al*., 1994). Many chemotherapeutic agents suppress growth of transformed or malignant cells by inducing apoptosis (Thompson, 1995; Hannun, 1997; Park *et al*., 1997). An induction of tumor cell apoptosis has become a major focus in the study of cancer therapy (White, 1996). The present study showed that pseudolaric acid B inhibits the cell growth and induces apoptosis in HeLa cells.

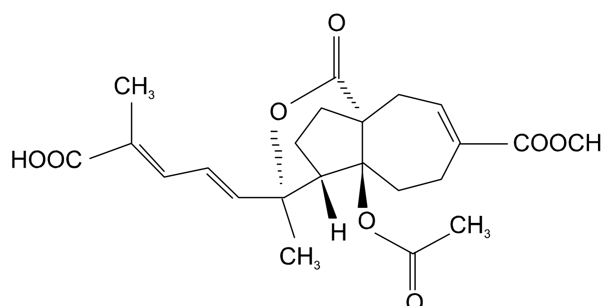


Figure 1. Chemical structure of pseudolaric acid B.

Materials and Methods

Materials

Pseudolaric acid B, purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Hoechst 33258, RNase A, Proteinase K, 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical (St. Louis, MO). Caspase-3 inhibitor benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethylketon e(z-DEVD-fmk), JNK MAPK inhibitor (SP600125) were from Calbiochem (San Diege, CA). Rabbit polyclonal antibodies against PARP, ICAD, p38, ERK, JNK, phospholated ERK, phospholated p38, phospholated JNK, mouse monoclonal antibodies against Bcl-2, Bcl-xL, and horseradish peroxidase-conjugated secondary antibodies (goat-anti-rabbit and goat-anti-mouse) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal anti-Bax was from Oncogene Research Products (Oncogene, MA).

Cell culture

HeLa, human cervical cells, were obtained from American Type Culture Collection (#CRL, 1872, Manassas, VA) and were cultured in RPMI-1640 medium (Hyclone, Logan, UT) supplemented with 10% heat inactivated (56°C, 30 min) fetal calf serum (Beijing Yuanheng Shengma Research Institution of Biotechnology, Beijing, China), 2 mM glutamine (Gibco, Grand Island, NY), and maintained at 37°C with 5% CO_2 in a humidified atmosphere.

Cell growth inhibition test

The inhibition of cultured cells was determined by MTT test. In brief, after incubation with pseudolaric acid B, HeLa cells (5×10^4/well) in 96-well plate were washed once with PBS and MTT (50 μg/100 μl of RPMI 1640) was added to each well. The cells were further incubated at 37°C for 4 h, and DMSO (150 μl) was added to dissolve the formazan crystals. Absorbance was measured at 492 nm with enzyme-linked immunosorbent assay plate reader (Tecan, Austria).

Observation of morphological changes by light microscopy

HeLa cells were treated with of pseudolaric acid B 5 μM for 24 h. Morphological changes were observed by phase contrast microscopy (Leica, Germany).

Nuclear damage observed by Hoechst 33258 staining

After treated by pseudolaric acid B, HeLa cells were collected by centrifugation at 1,000 *g* for 5 min, washed twice with PBS. The cells were fixed with 3.7% paraformaldelyde at room temperature for 2 h, centrifuged and washed with PBS, stained with Hoechst 33258 and fixed on glass slides, then observed with fluorescence microscopy (Nikon, Japan).

Determination of DNA fragmentation by agarose gel electrophoresis

HeLa cells, both adherent and floating, were collected by centrifugation at 1,000 *g* for 5 min. The cell pellet was suspended in cell lysis buffer (Tris-HCl 10 mM pH 7.4, edetic acid 10 mM pH 8.0, Triton-100 0.5%) and kept at 4°C for 30 min. The lysate was centrifuged at 25,000 *g* for 20 min. The supernatant was incubated with 20 mg/ml RNase A (2 μl) at 37°C for 1 h, then incubated with 20 mg/ml proteinase K (2 μl) at 37°C for 1 h. The supernatant was mixed with 5 M NaCl (20 μl) and isopropanol (120 μl) at -20°C overnight, then centrifuged at 25,000 *g* for 15 min. After drying, DNA was dissolved in TE buffer (Tris-HCl 10 mM pH 7.4, edetic acid 1 mM pH 8.0) and separated by 2% agarose gel electrophoresis at 100 V for 50 min.

Western blot analysis of protein expression

HeLa cells were treated with 5 μM pseudolaric acid B for indicated periods. Both adherent and floating cells were collected and frozen at -80°C. Western blot analysis was performed as follows. Briefly, the cell pellets were resuspended in lysis buffer, including 50 mM Hepes pH 7.4, 1% Triton-X 100, 2 mM sodium orthovanada, 100 mM sodium fluoride, 1 mM edetic acid 1 mM, egtazic acid, PMSF 1 mM, aprotinin 0.1 g/L, leupeptin 0.01 g/L, then lysed in 4°C for 1 h. After 13,000 *g* centrifugation for 10 min, the protein content of supernatant was determined using Bio-Rad protein assay reagent (Bio-Rad). 30 μg protein was loaded in each lane, then separated by 12% SDS polyacrylamide gel electrophoresis and blotted onto nitrocellulose membrane. Protein expression was detected using primary polyclonal antibody (1:200-1,000) and secondary polyclonal antibody (1:500) conjugated with peroxidase.

Results

Pseudolaric acid B induces apoptotic cell death in HeLa cells

To determine the inhibition of HeLa cells exposed to pseudolaric acid B, cells were treated with several doses of pseudolaric acid B, ranging from 0.16 to 100 μM for 12, 24, 36 and 48 h. The cytotoxicity of cells

was increased in a time- and dose-dependent manner (Figure 2).

Morphological changes were observed by phase contrast microscopy and fluorescence microscopy. By phase contrast microscopy, we observed a decrease in the total number of cells and an accumulation of cells floating in the culture medium after 24 h treatment with 5 μM pseudolaric acid B, indicating pseudolaric acid B-induced cell death (Figure 3B). Nuclear morphological changes were also observed by Hoechst 33258 staining. In control group, HeLa cells were round in shape and stained homogeneously. After 24 h treatment with 5 μM pseudolaric acid B, blebbing nuclei and granular apoptotic bodies appeared (Figure 3D). DNA fragmentation appeared obviously after 2.5 μM pseudolaric acid B treatment for 36 h on agarose gel electrophoresis (Figure 4).

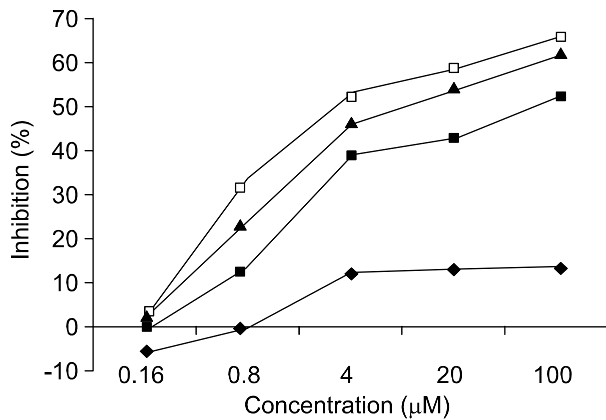


Figure 2. Inhibitory effect of pseudolaric acid B on HeLa cell growth. The cells (5×10^4 cells per well) were incubated with various concentrations of pseudolaric acid B for 12 h (◆), 24 h (■), 36 h (▲), and 48 h (□). Growth inhibition was evaluated by MTT method. $\bar{x} \pm s$, n = 3.

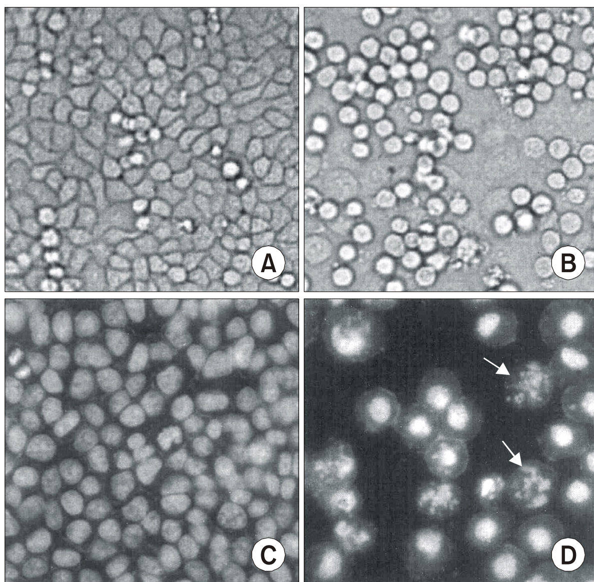


Figure 3. Morphological changes of HeLa cells by phase contrast microscopy and fluorescence microscope. A, C: control; B, D: Cells were treated with 5 μM pseudolaric acid B for 24 h. Arrows indicate condensed nuclei.

Effect of pseudolaric acid B on the expressions of MAPKs

MAPK signaling pathway has been shown to play an important role in cell growth and death. To determine whether MAPK cascade is involved in the pseudolaric acid B-induced cell death, Western blot analysis was performed to observe the expression of MAPKs. As shown in Figure 5A, the expression levels of ERK, p38 and JNK were not altered by pseudolaric acid B. However, expression of phosphorylated ERK was markedly decreased, whereas phosphorylated form of JNK was increased. The expression of phosphorylated p38 was not significant in its expression. The results suggested that ERK, JNK and p38 had different effects on pseudolaric acid B-induced HeLa cell apoptosis.

To further determine whether markedly increased expression of phosphorylated JNK participated in this apoptosis, specific inhibitor for JNK (SP600125) was applied to assess the function of JNK, The result showed that SP600125 significantly inhibited pseudolaric acid B induced growth inhibition at 24 h (Figure 5B).

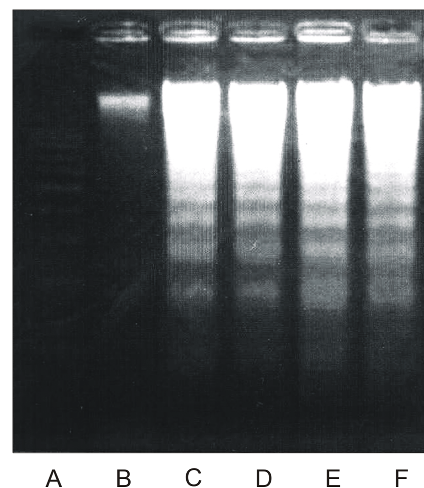
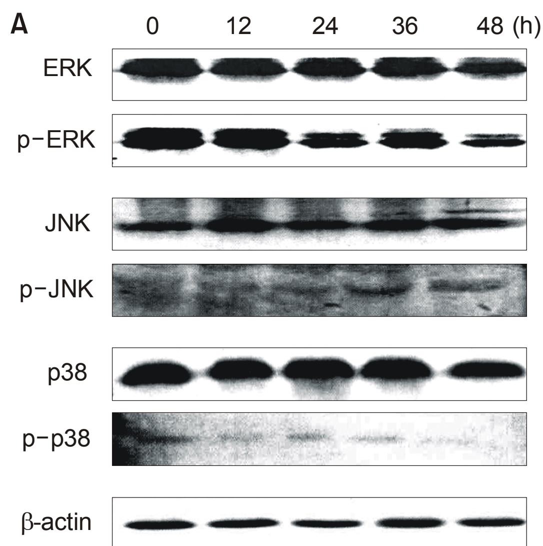


Figure 4. DNA fragmentation induced by pseudolaric acid B in HeLa cells. A: marker. B-F: HeLa cells were treated with pseudolaric acid B 0, 2.5, 5, 10, 20 μM for 36 h, respectively. DNA was isolated by agarose gel electrophoresis and analyzed by ethidium bromide staining.



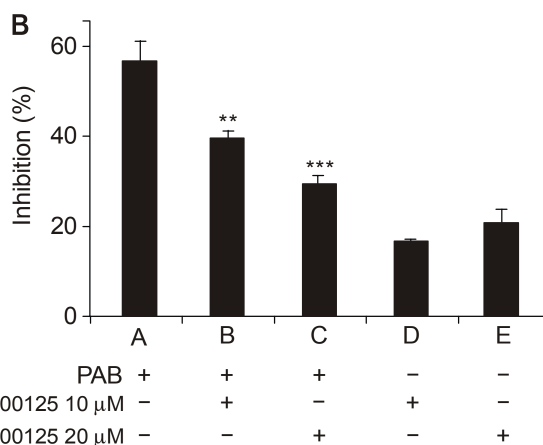


Figure 5. (A) The expression of MAPKs (ERK, JNK, p38) and phospholated MAPKs (phospholated ERK, phospholated JNK, phospholated p38) in 5 μM pseudolaric acid B-treated HeLa cells. (B) Effect of JNK inhibitor SP600125 on pseudolaric acid B-induced cell death. One hour prior to the addition of 5 μM pseudolaric acid B, HeLa cells were treated with JNK inhibitor SP600125 10 μM and 20 μM, then incubated for 24 h. The inhibition was measured by MTT method. $\bar{x} \pm s$, $n = 3$ (**$P < 0.01$, ***$P < 0.01$ *vs.* A group).

Effect of pseudolaric acid B on the expressions of Bcl-2, Bcl-x$_L$ and Bax

Mitochondrial Bcl-2 family is a series of proteins that regulate apoptosis. We measured the expressions of Bcl-2, Bcl-x$_L$ and Bax by Western blot analysis. After incubation with pseudolaric acid B, expression of Bcl-2 protein was decreased, and Bcl-x$_L$ did not change, whereas the level of Bax protein was increased in a time-dependent manner (Figure 6).

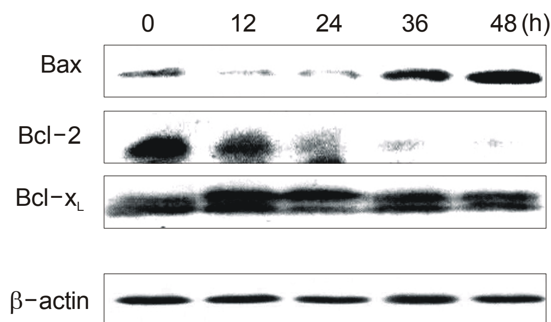
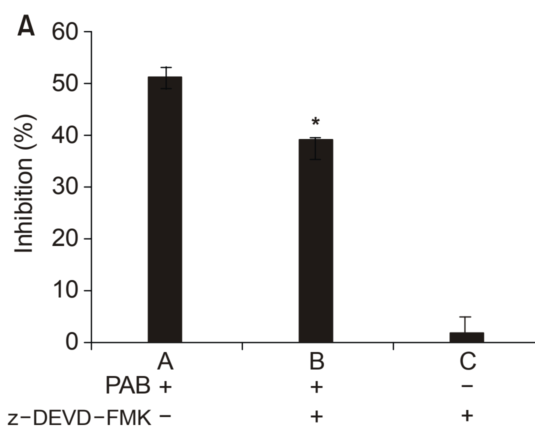


Figure 6. The expression of Bcl-2, Bcl-xL and Bax in 5 μM pseudolaric acid B-treated Hela cells.



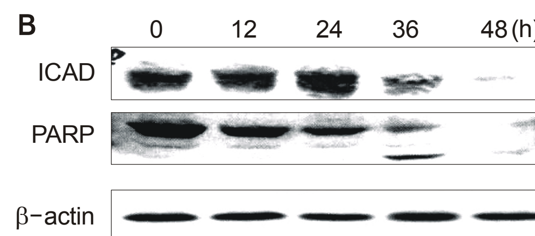


Figure 7. (A) Effect of caspase-3 inhibitor on pseudolaric acid B-induced HeLa cell apoptosis. The cells were cultured in the presence or absence of caspase-3 inhibitor. One hour prior to the addition of 5 μM pseudolaric acid B, z-DEVD-fmk (caspase-3 inhibitor, 10 μM) was added, then incubated for 24 h. $\bar{x} \pm s$, $n = 3$ (*$P < 0.05$ vs A group). (B) The expression of ICAD, PARP in 5 μM pseudolaric acid B-treated Hela cells.

Effect of caspase-3 on pseudolaric acid B-induced cytotoxicity in Hela cells

To investigate whether caspase-3 participated in pseudolaric acid B-induced cell apoptosis, HeLa cells were treated with 5 μM pseudolaric acid B for 24 h in the absence or presence of caspase-3 inhibitor, z-DEVD-fmk (20 μM). z-DEVD-fmk partially blocked

pseudolaric acid B-induced HeLa cell apoptosis (Figure 7A).

To further determine whether caspase-3 participates in pseudolaric acid B induced apoptosis in HeLa cells, Western blot analysis was performed to examine the expressions of caspase-3 substrates, PARP and ICAD. Figure 7B showed that the expression of minor PARP (85 kDa) protein after cleavage was up-regulated and ICAD protein expression was down-regulated by pseudolaric acid B, indicating that caspase-3 is activated. These results correspond to the activation of caspase-3 as shown in Figure 7A.

Discussion

Mitogen-activated protein kinases (MAPKs) are a family of serine/threonine kinases involved in the regulation of a wide range of cellular responses, including proliferation, differentiation, and survival of cells (Su and Karin, 1996). To date, several distinct MAP kinases have been identified to function in independent signaling pathways to affect the pleiotropic functions of this kinase family. These include p42/p44 extracellular signal- related kinases (ERK 1 and 2) (Boulton *et al*., 1991), c-Jun N-terminal protein kinase (JNK)/stress-activated protein kinase (SAPK) (Karakas *et al*., 1994), and p38 MAP kinase (Han *et al*., 1994). In general, the activation of p42/44 ERK activity inhibits apoptosis, while JNK/SAPK and p38 MAP kinases are associated with promotion of apoptosis.

In this study, pseudolaric acid B had no effects on the protein expression of ERK, JNK and p38 in HeLa cells, but the phosphorylation of ERK was down-regulated, whereas the phosphorylated JNK was markedly up-regulated. Furthermore, specific JNK inhibitor SP600125 significantly inhibited the cell death induced by pseudolaric acid B, indicating that JNK was involved in pseudolaric acid B-induced apoptosis in HeLa cells. A number of studies have indicated that activation of JNK plays a crucial role in apoptosis induced by various stimuli. For example, the JNK signaling pathway is essential for neuronal apoptosis in response to excitotoxic stress (Yang *et al*., 1997). Others have shown that UV-induced apoptosis in fibroblasts requires JNK for cytochrome c release from the mitochondria (Tournier *et al*., 2000). In our study, pretreatment of cells with SP600125 prevented pseudolaric acid B-induced apoptosis, suggesting involvement of JNK activation in this process. The expression of phosphorylated p38 was not obvious, suggesting that this pathway is unlikely to be involved in this cell apoptosis.

These results suggest that at early stages phosphorylated ERK protected cells from pseudolaric acid B-induced cell death, but at later stages, with the decrease of phosphorylated ERK, the participation of JNK in the cell death was more obvious.

The Bcl-2 family of proteins serve as critical regulators of pathways involved in apoptosis (Adams and Cory, 1998). The main protagonists are suggested to be anti-apoptotic Bcl-2 and pro-apototic Bax. If the concentration of Bcl-2 is enough to complex with at least half of Bax, then apoptosis is prevented (Burlacu, 2003).

HeLa cells treated with pseudolaric acid B exhibited elevated levels of Bax expression, while Bcl-2 was down-regulated. These results suggested that the mitochondrial pathway of cell death might be involved in pseudolaric acid B-induced HeLa cell death.

Caspases play an important role in apoptosis (Kwon *et al*., 2003). Caspases are activated during apoptosis, and cleave substrates such as PARP (Lazebnik *et al*., 1994), ICAD (Liu *et al*., 1997). PARP (116 kDa), a DNA repair enzyme, is probably best characterized caspase substrate, which is cleaved during apoptosis to a 24 kDa and a 85 kDa fragment representing the N-terminal DNA-binding domain and the C-terminal catalytic subunit, respectively. During apoptosis, PARP is selectively cleaved by several caspases, especially by caspase-3 (Kaufmann *et al*., 1993; Lazebnik *et al*., 1994). Detection of a 85 kDa or 24 kDa caspase cleavage fragment of PARP was shown to be a hallmark of apoptosis. ICAD is expressed as two forms, ICAD-L and ICAD-S. Both ICAD-L and ICAD-S carry two putative cleavage sites for caspase-3 (Sakahira *et al*., 1998). CAD exists as an inactive complex with ICAD. Caspases activated by apoptotic signals cleave ICAD to release CAD, which then enters the nucleus to degrade chromosomal DNA (Enari *et al*., 1998). In this paper, PARP (116 kDa) expression was down-regulated in a time dependent manner and minor 85 kDa fragment was increased, and ICAD was also down-regulated in a time dependent manner, which further confirmed that caspase-3 is activated in pseudolaric acid B induced cell apoptosis.

In conclusion, caspase cascade, ERK, JNK cascade, and mitochondrial pathway, involving Bax and Bcl-2, were involved in the pseudolaric acid B-induced HeLa cell apoptosis. The more detailed mechanism of pseudolaric acid B-induced HeLa cell apoptosis remains to be elucidated.

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