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Involvement of JNK-initiated p53 accumulation and phosphorylation of p53 in pseudolaric acid B induced cell death

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Abbreviations: ERK, extracellular signal-regulated protein kinase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; PKC, protein kinase C

Abstract

A terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay was used to determine that apoptosis causes HeLa cell death induced by pseudolaric acid B. The c-Jun N-terminal kinase (JNK) inhibitor SP600125 decreased p53 protein expression during exposure to pseudolaric acid B. SP600125 decreased the phosphorylation of p53 during pseudolaric acid B exposure, indicating that JNK mediates phosphorylation of p53 during the response to pseudolaric acid B. SP600125 reversed pseudolaric acid B-induced down-regulation of phosphorylated extracellular signal-regulated protein kinase (ERK), and protein kinase C (PKC) was activated by pseudolaric acid B, whereas staurosporine, calphostin C, and H7 partly blocked this effect. These results indicate that p53 is partially regulated by JNK in pseudolaric acid B-induced HeLa cell death and that PKC participates in pseudolaric acid B-induced HeLa cell death.

Keywords: apoptosis; HeLa cells; JNK mitogen-activated protein kinases; protein kinase C; pseudolaric acid B; tumor suppressor protein p53

Introduction

Pseudolaric acid B, a plant-derived diterpene acid isolated from *Pseudolarix kaempferi Gordon* (*Pinaceae*), has various physiological and pharmacological effects such as antifungal, antimicrobial (Li *et al.*, 1995), antifertility (Wang *et al.*, 1982; 1988), and cytotoxic activity (Pan *et al.*, 1990). Our previous studies showed that pseudolaric acid B had cytotoxic activity against human cervical carcinoma HeLa cells (Gong *et al.*, 2004) and human melanoma A375-S2 cells (Gong *et al.*, 2005). However, the precise mechanism of this antitumor effect remains unclear.

Mitogen-activated protein kinases (MAPKs), such as extracellular signal-regulated kinase (ERK), the c-jun N-terminal kinase (JNK), and p38 MAPK are involved in signal transduction and regulation of cellular process including proliferation, cell growth, differentiation, and apoptosis (Brunet *et al.*, 1999; Schaeffer *et al.*,1999; Johnson *et al.*, 2002). ERK is involved in the control of cell growth and differentiation. In contrast, activation of JNK and p38 MAPK is associated with induction of apoptosis (Cross *et al.*, 2000; Kowan *et al.*, 2003).

p53 is a tumor suppressor gene that is mutated in more than 50% of all human tumors (Levine, 1997). Designing efficient ways to restore wild p53 is, therefore, one of the key issues in cancer research. Activated p53 may signal cells to undergo growth arrest, cell differentiation, or apoptosis. p53 has been described as 'the guardian of the genome', referring to its role in conserving stability by preventing genome mutation (Levine, 1997).

Protein kinase C (PKC) is a multigene family of phospholipid-dependent serine-threonine kinases that plays a central role in signal transduction and has been implicated in a wide range of physiological and abnormal cellular functions, such as cell growth, transformation, and differentiation. The twelve members of the PKC superfamily that are known so far are divided into three groups based on their requirements for activation. The existence of this large family of PKC isotypes indicates that individual PKC isotypes likely have specific roles in signal transduction (Besson *et al.*, 2000).

In this study, we investigated the involvement of JNK and its relationship with p53 in pseudolaric acid B-treated HeLa cells. Pseudolaric acid B strongly stimulated p53, while the JNK inhibitor SP600125

markedly reversed this process. Up-regulation of PKC also contributed to pseudolaric acid B-induced cell apoptosis.

Materials and Methods

Chemicals

Pseudolaric acid B was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). RNase A, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), proteinase K, Hoechst 33258, staurosporine, calphostin C, and H7 were purchased from Sigma Chemical (St. Louis, MO). JNK MAPK inhibitor (SP600125) was obtained from Calbiochem (San Diego, CA). A TACS^{TM}2 TdT-DAB *In Situ* Apoptosis Detection Kit was a product of Trevigen (Gaitherburg, MD). A PKC activity assay kit was obtained from Promega (Madison, WI). Rabbit polyclonal antibodies against ERK, JNK, phosphorylated-ERK, phosphorylated-JNK, and phosphorylated-p53, mouse monoclonal antibody against p53, and horseradish peroxidase-conjugated secondary antibodies (goat-anti-rabbit and goat-anti-mouse) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell culture

HeLa, human cervical cells were obtained from

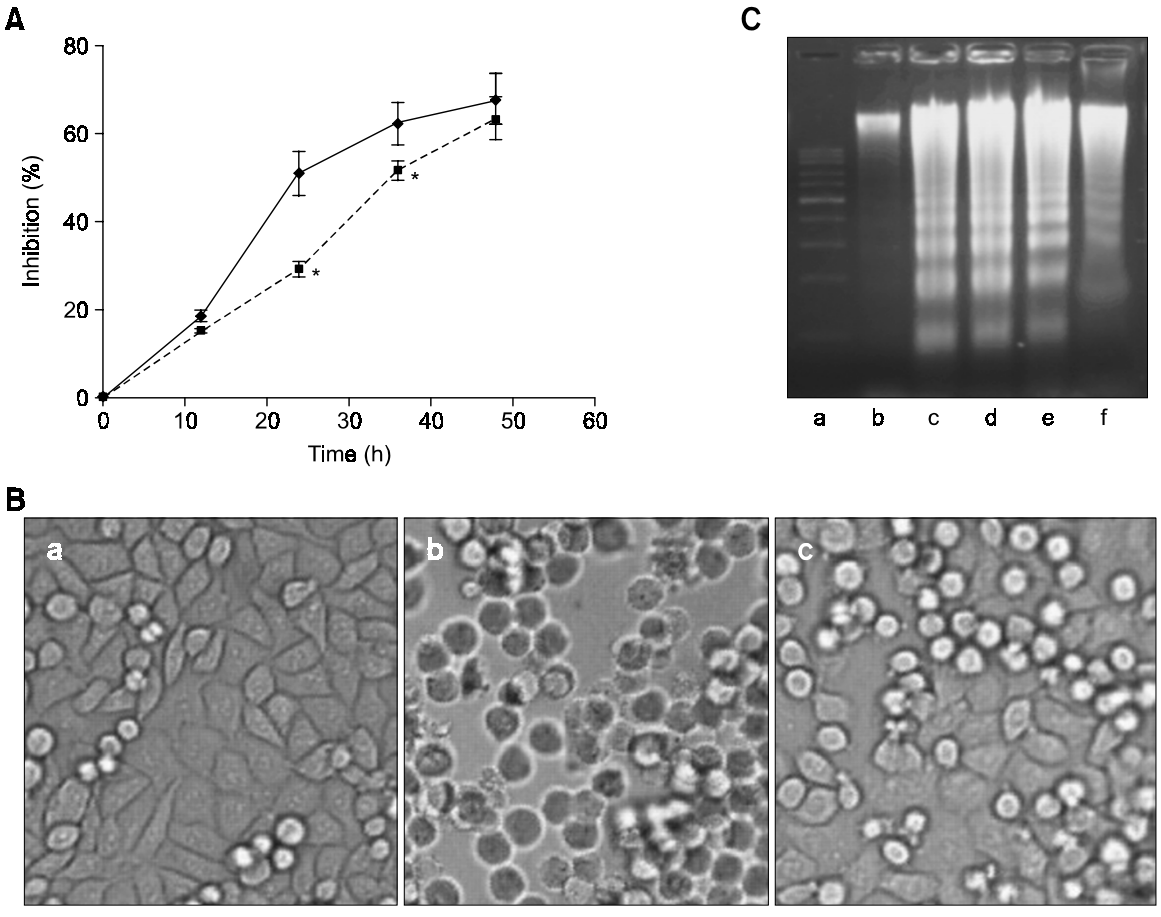

Figure 1. Effect of the JNK inhibitor SP600125 on pseudolaric acid B-induced apoptosis. One hour prior to addition of 5 μM pseudolaric acid B, HeLa cells were treated with SP600125 (20 μM), then further incubated for the indicated time periods. (A) Cell death was measured using an MTT assay (–◆–, pseudolaric acid B treated group; –■–, pseudolaric acid B and SP600125 treated group). $n = 3$. Mean ± SD * $P < 0.01$ *vs.* pseudolaric acid B treated group. (B) Morphological changes were observed under phase contrast microscopy at 24 h (a, control; b, 5 μM pseudolaric acid B-treated group; c, 5 μM pseudolaric acid B and SP600125 treated group, × 200). (C) DNA fragmentation induced by pseudolaric acid B and blockage of pseudolaric acid B induced DNA fragmentation by SP600125 in HeLa cells. a, DNA molecular weight marker; b-e, HeLa cells were incubated with 0, 1.2, 2.5, 5 μM pseudolaric acid B for 36 h; f, HeLa cells were pretreated with 20 μM SP600125 for 1h, then incubated with 5 μM pseudolaric acid B.

American Type Culture Collection (Manassas, VA) and 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) and 2 mM glutamine (Gibco, Grand Island, NY). Cultures were maintained at 37°C under a 5% CO_2 humidified atmosphere.

Assessment of apoptosis

Apoptotic cells were assessed by terminal deoxynucleotidyltransferase-mediated dUTP end labeling (TUNEL) and DNA fragmentation.

Cell growth inhibition test

The cytotoxicity of pseudolaric acid B in HeLa cells was analyzed using an MTT test. In brief, after addition of MTT (0.5 mg/ml), cells (5×10^4 cells/well) were incubated at 37°C for 4 h. DMSO (150 μl) was added to dissolve formazan crystals. The absorbance was measured at 492 nm using an enzyme-linked immunosorbent assay plate reader (Tecan, Salzburg, Austria).

Observation of morphological changes

HeLa cells were treated with 5 μM pseudolaric acid B in the presence or absence of 20 μM SP600125 or 40 nM staurosporine for indicated time periods. Morphological changes were observed under phase contrast microscopy (Leica, Wetzlar, Germany).

TUNEL assay

A TUNEL assay was used for detection of DNA strand breaks. Detection was carried out according to the instructions for the TACS™2 TdT-DAB *In Situ* Apoptosis Detection Kit. Briefly, the cells were rinsed once with PBS and fixed in 3.7% buffered formaldehyde at room temperature for 10 min. The fixed sections were pretreated with 10% H_2O_2, and end-labeling was performed using a TdT labeling

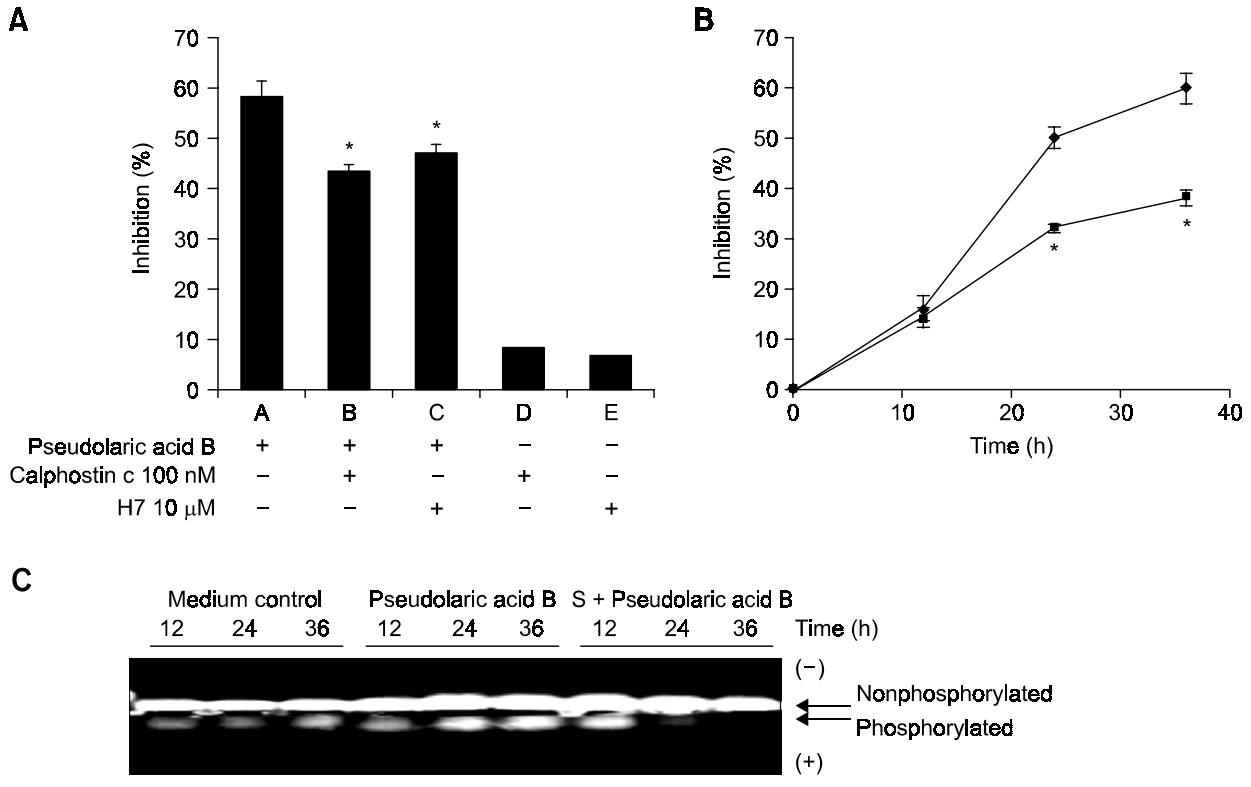

Figure 2. Effect of PKC on pseudolaric acid B-induced apoptosis. (A) One hour prior to addition of 5 μM pseudolaric acid B, HeLa cells were treated with calphostin C (100 nM) or H7 (10 μM), then incubated for 24 h. Cell death was measured using an MTT assay. $n = 3$. Mean ± SD * $P < 0.01$ *vs.* pseudolaric acid B treated group. (B) One hour prior to addition of 5 μM pseudolaric acid B, HeLa cells were treated with staurosporine (40 nM), then further incubated for the indicated time periods. Cell death was measured using an MTT assay (–◆–, pseudolaric acid B treated group; –■–, pseudolaric acid B and staurosporine treated group). $n = 3$. Mean ± SD * $P < 0.01$ *vs.* pseudolaric acid B treated group. (C) Effects of pseudolaric acid B on PKC activity in HeLa cells. The cells were pretreated with 40 nM staurosporine (S) for 1 h, incubated with 5 μM pseudolaric acid B for indicated time periods, then the PKC activity was measured.

reaction mix at 37°C for 1 h. Nuclei exhibiting DNA fragmentation were visualized by incubation in 3,3'-diamino benzidine (DAB) for 7 min. Lastly, the sections were counterstained with methyl green and observed under light microscopy. The nuclei of apoptotic cells were stained dark brown, and TUNEL-positive cells were determined by randomly counting 100 cells.

DNA fragmentation assay

HeLa cells were collected by centrifugation at 1,000 × *g* for 5 min. The cell pellet was suspended in a cell lysis buffer (10 mM Tris-HCl pH 7.4, 10 mM EDTA, pH 8.0, Triton X-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 of RNase A (2 μl) at 37°C for 1 h, then incubated with 20 mg/ml of 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 (10 mM Tris-HCl pH 7.4, and 1 mM EDTA, pH 8.0) and separated by 2% agarose gel electrophoresis at 100 V for 50 min.

PKC activity assay

A PKC activity assay was carried out according to the instructions for the PepTag® Non-Radioactive Protein Kinase C Assay Kit. Briefly, the cells were washed once with cold PBS and lysed on ice in cold lysis buffer, including 20 mM Tris-HCl, 0.5 mM EGTA, 2 mM EDTA, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 10 mg/ml of leupeptin (pH 7.5). Assays were then performed at 30°C in a total volume of 25 μl containing 5 μl of PKC reaction 5 × buffer, 5 μl of PLSRTLSVAAK peptide, 5 μl of PKC activator, 1 μl of peptide protection solution, and 9 μl of sample. Reactions were initiated by addition of 9 μl of sample and terminated after 30 min by incubation of the reaction mixture at 95°C for 10 min. After adding 1 μl of 80% glycerol, each sample was separated by 0.8% agarose gel electrophoresis at 100 V for 15 min. Phosphorylated peptide migrated toward the anode (+), while nonphosphorylated peptide migrated toward the cathode (-).

Western blot analysis

HeLa cells were treated with 5 μM pseudolaric acid B for indicated time periods. Both adherent and floating cells were collected and frozen at -80°C. Western blot analysis was then performed. Briefly, the cell pellets were resuspended in a lysis buffer, including 50 mM Hepes, pH 7.4, 1% Triton-X 100, 2 mM sodium orthovanada, 100 mM sodium fluoride, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 100 μg/ml of aprotinin, and 10 μg/ml of leupeptin, then lysed at 4°C for 1 h. After 13,000 × *g* centrifugation for 10 min, the protein content of the supernatant was determined using a Bio-Rad protein assay reagent (Hercules, CA). The protein lysates were separated by 12% SDS polyacrylamide gel electrophoresis and blotted onto nitrocellulose membrane. Protein expression was detected using primary polyclonal antibodies and secondary polyclonal antibodies conjugated with horseradish peroxidase.

Results

Induction of apoptosis by pseudolaric acid B

Pseudolaric acid B induced HeLa cell death in a time- and dose-dependent manner (Gong *et al.*, 2004). To determine the characteristics of HeLa cell death, a TUNEL assay was carried out. In the control group, the ratio of TUNEL-positive cells was 9.3 ± 2.5%. In the presence of 2.5 and 5 μM pseudolaric acid B, the numbers of apoptotic cells increased to 20.7 ± 3.8 and 34.7 ± 3.0% at 24 h, respectively (Table 1). These results demonstrated that apoptosis was a cause of HeLa cell death induced by pseudolaric acid B.

Inhibitory effects of SP600125 on pseudolaric acid B-induced morphologic changes and DNA fragmentation

Pseudolaric acid B-induced HeLa cell death was partially inhibited by the JNK inhibitor SP600125 (Figure 1A). In order to further determine the participation of JNK in cell death, the effects of the JNK inhibitor SP600125 on cell morphological changes and DNA fragmentation were examined. SP600125 inhibited pseudolaric acid B-induced HeLa cell death (Figure 1B) and DNA fragmentation (Figure 1C).

Table 1. Quantitative analysis of TUNEL-positive HeLa cells.

Pseudolaric acid B (μM)	Apoptotic cells (%) (TUNEL-positive)
0	9.3 ± 2.5
2.5	20.7 ± 3.8*
5.0	34.7 ± 3.0**

The cells were treated with pseudolaric acid B for 24 h. The results are representative of three independent experiments. All data are presented as mean ± SD and analyzed for statistical significance using Student's *t*-test. *P* values of less than 0.05 were considered to be significant (*$P < 0.05$, **$P < 0.01$). *P* values are compared with the 0 μM group.

Activation of PKC in pseudolaric acid B-induced cell

Since the PKC inhibitor, staurosporine, calphostin C, and H7 all markedly inhibited pseudolaric acid B-induced cell death (Figure 2A and Figure 2B), the PKC activity was measured. The PKC activity was increased by treatment with 5 μM pseudolaric acid B at 24 h and persistently activated to 36 h. The PKC inhibitor, staurosporine inhibited PKC activity (Figure 2C).

Effects of SP600125 on protein expression and phosphorylation of p53 in pseudolaric acid B-treated HeLa cells

p53 was activated in pseudolaric acid B-treated HeLa cells. To verify whether JNK activation was required in pseudolaric acid B-induced p53 activation, HeLa cells were incubated in the presence of SP600125 for indicated time periods. p53 expression and p53 phosphorylation in response to pseudolaric acid B were partially blocked by SP600125. Pretreatment with SP600125 effectively reversed pseudolaric acid B-induced down-regulation of ERK phosphorylation from 24 to 36 h (Figure 3).

Discussion

JNK is one of the key mediators activated by exposure to cytokines and environmental stresses that regulate inflammatory responses, cell growth, proliferation, survival, and apoptosis (Ip *et al.*, 1998; Davis *et al.*, 2000). In this study, we demonstrated that JNK plays an important role in pseudolaric acid B-induced cell death. Inhibition of JNK by the specific JNK inhibitor SP600125 inhibited pseudolaric acid B-induced cell death, changes in cellular morphology, and DNA fragmentation induced by pseudolaric acid B.

The prevention of cancer is profoundly dependent on the p53 tumor suppressor protein. The ability of p53 to eliminate excess, damaged, or infected cells by apoptosis is vital for the proper regulation of cell proliferation in multi-cellular organisms (Haupt *et al.*, 2003). p53 has been referred to as the 'gatekeeper' of genomic stability because of the important role it plays in DNA damage recognition, growth arrest, and apoptosis (Brooks *et al.*, 2003). Our results showed that after 24 h of exposure to pseudolaric acid B the amount of the p53 protein began to increase and, simultaneously, the amount of phosphorylated-p53 also increased, indicating that the p53 protein participates in this process.

It has been reported that p53 is also a JNK substrate (Milne *et al.*, 1995; Fuchs *et al.,* 1998a, b). JNK plays two distinct roles in the control of p53 activity. When inactivated, this kinase binds to residues 97-116 in p53 and targets p53 for degradation by proteasome. Expression of a cons-

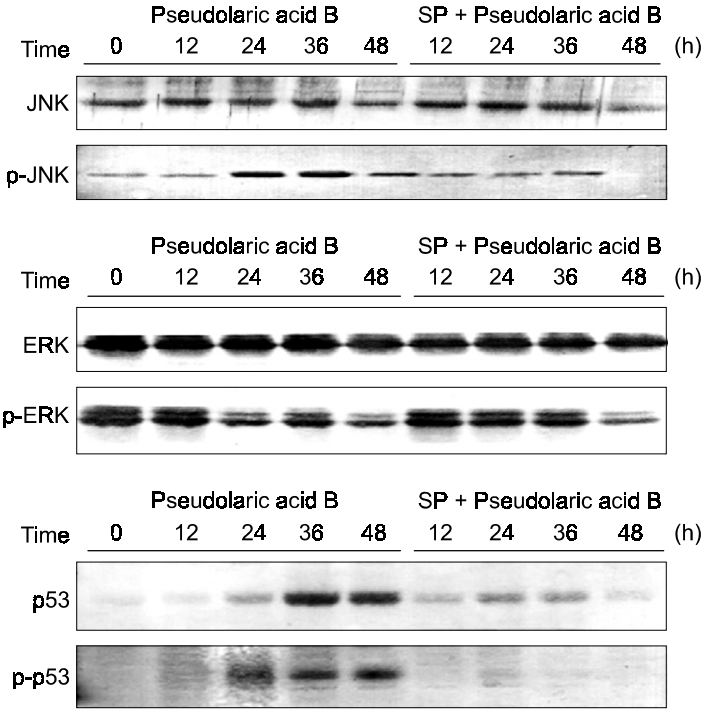

Figure 3. Effect of the JNK inhibitor SP600125 on the change of protein expression induced by pseudolaric acid-B. The cells were pretreated with 20 μM SP600125 (SP) for one hour, followed by administration of 5 μM pseudolaric acid B for the indicated time periods. Protein expression was determined by western blot analysis.

titutively activated JNK, or activation of the upstream kinase MEKK1, increases the level of p53 by protection from degradation (Fuchs *et al.,* 1998b). In contrast, active JNK phosphorylates p53 on threonine 81 and participates in its activation (Buschmann *et al.*, 2001). In this study, the JNK specific inhibitor SP600125 reversed pseudolaric acid B-induced p53 activation. Pseudolaric acid B-induced phosphorylated-ERK activation was also reversed. ERK exerts a protective function against apoptosis induced by growth factor deprivation, and the dynamic balance between the ERK and JNK pathways may be important in determining cell survival (Cross *et al.*, 2000). SP600125 increased ERK phosphorylation and decreased JNK phosphorylation, which contributed to the survival of cells. Our results indicated that p53 lies downstream of JNK, and that inhibition of ERK activation was also involved in pseudolaric acid B-induced cell death.

PKC is an attractive target for modulation of apoptosis as there is mounting evidence that PKC is a multifaceted regulator of cellular sensitivity to chemotherapeutic agents. PKC has been shown to activate the ERK pathway (Schonwasser *et al.*, 1998). Activation of a certain PKC isozyme has also been shown to regulate T cell activation and macrophage apoptosis through a JNK-dependent pathway (Werlen *et al.*, 1998; Castrillo *et al.*, 2003; Comalada *et al.*, 2003).

In our study, staurosporine, calphostin C, and H7 markedly inhibited pseudolaric acid B-induced cell death, indicating PKC participation in this process. PKC activity was increased at 24 h after pseudolaric acid B administration and was maintained to 36 h, demonstrating that PKC participated in pseudolaric acid B-induced cell death.

JNK-induced activation of p53 was involved in pseudolaric acid B-induced apoptosis in HeLa cells. Simultaneously, activation of PKC also contributed to pseudolaric acid B-induced apoptosis. More details remains to be elucidated.

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