

Full-length article

Natural jasmonates of different structures suppress the growth of human neuroblastoma cell line SH-SY5Y and its mechanisms¹Qiang-song TONG^{2,5}, Guo-song JIANG², Li-duan ZHENG³, Shao-tao TANG², Jia-bin CAI², Yuan LIU², Fu-qing ZENG², Ji-hua DONG⁴²Departments of Surgery, ³Pathology and ⁴Central Laboratory, Huazhong University of Science and Technology, Wuhan 430022, China**Key words**

jasmonate; neuroblastoma; proliferating cell nuclear antigen; N-myc; X-linked inhibitor of apoptosis protein; survivin

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Abstract

Aim: Recent evidence has indicated that members of natural jasmonates, a family of plant stress hormones, exhibit anticancer activity. The current study was undertaken to investigate the effects of jasmonates on the *in vitro* growth of human neuroblastomas, one of the most common solid tumors in children. **Methods:** Cellular proliferation was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide colorimetry and colony formation assay. Apoptosis was detected by Hoechst 33258 staining and flow cytometry. Western blotting was applied to assay gene expression. **Results:** The administration of natural jasmonates, methyl jasmonate, *cis*-jasmone, and jasmonic acid to cultured neuroblastoma cell line SH-SY5Y, resulted in a decrease of cell proliferation in a dose- and time-dependent manner. However, the *in vitro* growth of cultured human embryonic kidney (HEK) cell line HEK 293 was not affected by jasmonates. The cell cycles of jasmonate-treated SH-SY5Y cells were arrested at the G₂/M phase. The incubation of SH-SY5Y cells with jasmonates resulted in characteristic changes of apoptosis. The anticancer activities of natural jasmonates on SH-SY5Y cells are as follows: methyl jasmonate > *cis*-jasmone > jasmonic acid. In addition, the expressions of proliferating cell nuclear antigen and N-myc were downregulated by methyl jasmonate. Moreover, methyl jasmonate decreased the expression of the X-linked inhibitor of apoptosis protein and survivin, critical members of inhibitors of the apoptosis protein family, in SH-SY5Y cells. **Conclusion:** Jasmonates suppress the growth of human neuroblastoma cell line SH-SY5Y via inhibiting cell proliferation and inducing apoptosis, which lays the groundwork for further investigation into the anticancer activities and its mechanisms of natural jasmonates on human neuroblastomas.

Introduction

Neuroblastomas are a highly malignant tumor derived from neural crest cells and account for approximately 10% of all childhood malignancies and 50% of childhood cancer deaths^[1]. During recent years, the molecular mechanisms that control neuroblastoma development and progression have been well understood, such as the amplification of N-myc proto-oncogene, the deletion of chromosome 1p, and the complete or partial gain of the long arm of chromosome 17^[2]. However, despite treatment regimens, including radia-

tion therapy and high-dose chemotherapy with stem cell rescue, little progress has been made in improving the poor prognosis of patients with late-stage neuroblastomas because resistance to chemotherapy is common^[3]. Therefore, new anticancer substances and treatment regimens are of interest.

In recent years, several groups have reported that members of jasmonates, a family of plant stress hormones, and some of their synthetic derivatives, exhibit anticancer activity *in vitro* and *in vivo*^[4]. A wide spectrum of malignancies,

including the most common human cancers, such as prostate cancer, breast cancer, and lung cancer, exhibited sensitivity to the cytotoxic effects of jasmonates^[5,6]. The administration of methyl jasmonate per os daily, significantly increased the survival of lymphoma-bearing C57BL mice^[5]. However, it still remains largely unknown whether jasmonates exert effects on the proliferation and apoptosis of human neuroblastoma cells. In the present study, we demonstrated for the first time that the most common natural jasmonates, methyl jasmonate, *cis*-jasmone, and jasmonic acid suppress the growth of cultured neuroblastoma cells. The efficiency of jasmonates was found to be correlative with their chemical structures. In addition, the anticancer effects of jasmonates on human neuroblastoma cells were associated with the downregulation of proliferating cell nuclear antigen (PCNA) and N-myc, and the modulation of the expression of the X-linked inhibitor of apoptosis protein (XIAP) and survivin, critical members of the inhibitor of the apoptosis protein (IAP) family.

Materials and methods

Cell culture and jasmonate treatment Human neuroblastoma cell line SH-SY5Y and human embryonic kidney cell line (HEK) 293 were purchased from American Type Culture Collection (USA) and grown in minimal essential medium (Life Technologies, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS; Life Technologies, USA), penicillin (100 U/mL), and streptomycin (100 µg/mL). The cells were maintained at 37 °C in a humidified atmosphere of 5% CO₂. Confluent monolayers of cells were incubated with different concentrations of methyl jasmonate, *cis*-jasmone, jasmonic acid, cisplatin, or DMSO (all from Sigma, St Louis, MO, USA) as indicated.

Measurement of cell proliferation Confluent monolayers of SH-SY5Y and HEK 293 were trypsinized, and 4×10³ viable cells suspended in 0.1 mL culture medium supplemented with 10% FBS were seeded onto each well of 96-well plates. After overnight an incubation to allow cell attachment, the cells were incubated with jasmonates (0.5, 1.5, and 2.5 mmol·L⁻¹), cisplatin (0.025, 0.05, and 0.1 mmol·L⁻¹) or DMSO for 6, 12, and 24 h. At the end of the incubation period, cell proliferation was monitored by the 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT; Sigma, USA) colorimetric assay^[7]. Briefly, 20 µL MTT (5 mg/mL) was added to each well. After 4 h of incubation at 37 °C, the cell supernatants were discarded, blue precipitate was dissolved with DMSO, and the absorbance was measured at 570 nm (*A*_{570 nm}). Cellular proliferation inhibition rates were calculated as follows: (%)=(1-average *A*_{570 nm} value of treated cells/aver-

age *A*_{570 nm} value of untreated control cells)×100%. The 50% inhibitory concentration (IC₅₀) of 24 h exposure, defined as the drug concentration resulting in a 50% reduction of cell proliferation compared to untreated control cells, was determined by Bliss software (Bliss, CA, USA). All experiments were performed with 6 wells for each concentration, and at least 3 independent tests were performed.

Colony formation assay The cells were seeded at a density of 500/mL on 35 mm dishes^[8]. After an overnight incubation to allow cell attachment, the cells were incubated with natural jasmonates (2.5 mmol·L⁻¹), cisplatin (0.025 mmol·L⁻¹), or DMSO as indicated. After incubation for 24 h, the medium was replaced with fresh medium containing 10% FBS. Colonies were allowed to grow for 10-14 d. The medium was discarded and each well was carefully washed twice with phosphate-buffered saline (PBS). The cells were fixed in methanol for 15 min and then stained with crystal violet for 20 min. Finally, positive colony formations (more than 50 cells/colony) were counted. The survival fraction for cells was expressed as the ratio of plating efficiency of treated cells to that of untreated control cells.

Cell cycle assay According to the literature^[9], cell cycles in untreated, DMSO-, natural jasmonate- (0.5, 1.5, and 2.5 mmol·L⁻¹), or cisplatin (0.025 mmol·L⁻¹)-treated cells were examined by flow cytometry. Briefly, 2×10⁵ cells were collected, washed twice with 0.01 mol·L⁻¹ PBS, and fixed in 70% ethanol overnight at 4 °C. The cells were then washed once with PBS, digested by 200 µL RNase (1 mg/mL) at 37 °C for 30 min, and stained with 800 µL propidium iodide (PI; 50 µg/mL, Sigma, USA) at room temperature for 30 min. The DNA histograms were assayed with a flow cytometer (Becton Dickinson, San Jose, CA, USA), using CELLQUEST software (Becton Dickinson, USA).

Cellular morphological observation To observe the changes in cellular morphology, Hoechst 33258 (Sigma, USA) staining was performed^[10]. Briefly, cells were incubated with natural jasmonates (2.5 mmol·L⁻¹), cisplatin (0.025 mmol·L⁻¹), or DMSO as indicated, and fixed with 4% formaldehyde in PBS solution at 4 °C for 10 min. After 3 washes with PBS, the cells were stained with 10 mg/L Hoechst 33258 in PBS solution at 4 °C in the dark for 10 min, and morphological changes, including cell shrinkage and nuclear condensation, were observed under a fluorescence microscope (Olympus AX80; Olympus, Tokyo, Japan).

Detection of apoptosis Apoptotic ratios of cells were determined by Annexin V-fluorescein isothiocyanate (FITC; BD Pharmingen, San Diego, CA, USA) and PI staining flow cytometry^[11]. Briefly, the cells treated with natural jasmonates (2.5 mmol·L⁻¹), cisplatin (0.025 mmol·L⁻¹), or DMSO were

collected, washed twice with cold PBS, resuspended in 100 μL binding buffer (10 $\text{mmol}\cdot\text{L}^{-1}$ HEPES, 140 $\text{mmol}\cdot\text{L}^{-1}$ NaCl, and 2.5 $\text{mmol}\cdot\text{L}^{-1}$ CaCl_2 , pH 7.4) into 2×10^5 – 5×10^5 cells/mL density, and incubated with Annexin V–FITC at room temperature for 10 min. After washing with binding buffer, the cells were resuspended in 400 μL binding buffer containing 10 μL PI (20 $\mu\text{g}/\text{mL}$), and incubated on ice for 15 min. Apoptosis was analyzed by a flow cytometer (Becton Dickinson, USA) at a wavelength of 488 nm. This method can be used to distinguish living cells (Annexin V⁻/PI⁻), early apoptotic/primary apoptotic cells (Annexin V⁺/PI⁻), late apoptotic/secondary necrotic cells (Annexin V⁺/PI⁺), and necrotic cells (Annexin V⁻/PI⁺)^[11].

Western blotting for PCNA, N-myc, XIAP, and survivin Cells treated with methyl jasmonate (0.5, 1.5, and 2.5 $\text{mmol}\cdot\text{L}^{-1}$), cisplatin (0.025 $\text{mmol}\cdot\text{L}^{-1}$), or DMSO were collected and extracted with 1 \times cell lysis buffer (Promega, Madison, WI, USA). Proteins (50 μg) from each sample were subjected to 4%–20% precast polyacrylamide gel (Bio-Rad, Hercules, CA, USA) electrophoresis and transferred to nitrocellulose membranes (Bio-Rad, USA). For PCNA, N-myc, XIAP, and survivin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) detections, the primary antibody dilutions were 1:1000, 1:500, 1:500, and 1:500, respectively, followed by 1:3000 dilution of goat antirabbit horseradish peroxidase-labeled antibody (Bio-Rad, USA). The ECL substrate kit (Amersham, Piscataway, NJ, USA) was used for the chemiluminescent detection of signals with autoradiography film (Amersham, USA).

Statistical analysis Unless otherwise stated, all data were shown as mean \pm SEM. Statistical significance ($P<0.05$) was determined by *t*-test or ANOVA followed by an assessment of differences using SPSS 12.0 software (SPSS, Chicago, IL, USA).

Results

Natural jasmonates of different structures suppress the growth of SH-SY5Y cells To explore the effects of natural jasmonates (Figure 1) on human neuroblastoma cells, we first observed the cell proliferation changes in cultured SH-SY5Y cells. As shown in Figure 2A, the MTT colorimetric assay indicated that methyl jasmonate (0.5, 1.5, and 2.5 $\text{mmol}\cdot\text{L}^{-1}$), *cis*-jasmone (0.5, 1.5, and 2.5 $\text{mmol}\cdot\text{L}^{-1}$), jasmonic acid (0.5, 1.5, and 2.5 $\text{mmol}\cdot\text{L}^{-1}$), and cisplatin (0.025, 0.05, and 0.1 $\text{mmol}\cdot\text{L}^{-1}$), but not DMSO, inhibited the cell proliferation of SH-SY5Y cells in a dose- and time-dependent manner. The incubation of SH-SY5Y cells with 2.5 $\text{mmol}\cdot\text{L}^{-1}$ methyl jasmonate, *cis*-jasmone, and jasmonic acid, and 0.1 $\text{mmol}\cdot\text{L}^{-1}$ cisplatin, resulted in a decrease of cell proliferation by 81.7%, 48.9%, 12.65%,

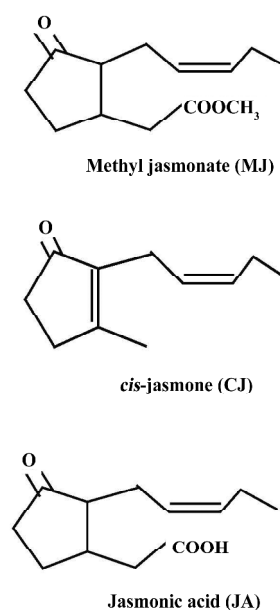


Figure 1. Natural jasmonates structures. Three of the most common natural jasmonates, methyl jasmonate, *cis*-jasmone, and jasmonic acid, were involved in this study, and differences in their chemical structures were compared.

and 86.4%, respectively. The IC_{50} values of the SH-SY5Y cells treated with methyl jasmonate, *cis*-jasmone, and jasmonic acid for 24 h were 1.65, 3.33, and 556.8 $\text{mmol}\cdot\text{L}^{-1}$, respectively, which were higher than that of cisplatin (0.025 $\text{mmol}\cdot\text{L}^{-1}$) as a positive control. Moreover, cisplatin (0.025, 0.05, and 0.1 $\text{mmol}\cdot\text{L}^{-1}$), but not methyl jasmonate, *cis*-jasmone, jasmonic acid, or DMSO, suppressed the cell proliferation of the HEK 293 cell line by 46.8%–72.6% (Figure 2B). The colony formation assay further demonstrated that the administration of natural jasmonates and cisplatin, but not DMSO, resulted in the decreased cell proliferation of SH-SY5Y cells (Figure 3). The administration of 2.5 $\text{mmol}\cdot\text{L}^{-1}$ methyl jasmonate, *cis*-jasmone, and jasmonic acid resulted in the loss of colony formation capability of SH-SY5Y cells by 87.6%, 64.1%, and 21.2%, respectively.

Natural jasmonates induce cell cycle arrest and apoptosis in SH-SY5Y cells As previous studies have demonstrated that inducing apoptosis is one of the mechanisms of the anticancer effects of jasmonates^[5,6,12], we hypothesized that natural jasmonates may also induce apoptosis in cultured neuroblastoma SH-SY5Y cells. The cell cycle assay showed that methyl jasmonate and *cis*-jasmone, but not jasmonic acid, induced G₂/M phase arrest in cultured SH-SY5Y cells (Table 1). As shown in Figure 4A, Hoechst 33258 staining indicated that the administration of methyl jasmonate (2.5 $\text{mmol}\cdot\text{L}^{-1}$), *cis*-jasmone (2.5 $\text{mmol}\cdot\text{L}^{-1}$), jasmonic acid (2.5 $\text{mmol}\cdot\text{L}^{-1}$), and cisplatin (0.025 $\text{mmol}\cdot\text{L}^{-1}$), rather than DMSO,

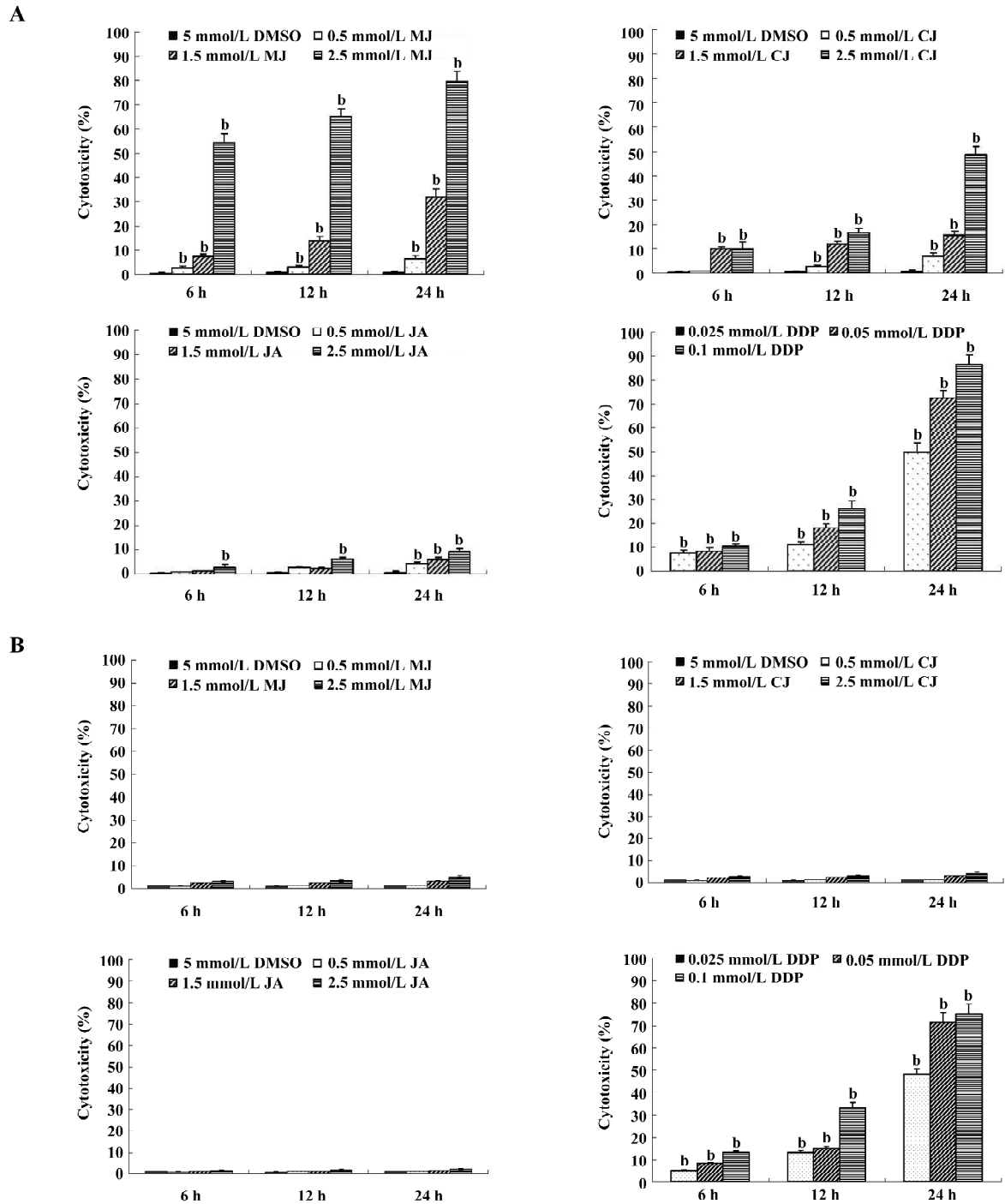


Figure 2. Natural jasmonates suppress the proliferation of SH-SY5Y cells and HEK 293 cells. Confluent monolayers of human neuroblastoma SH-SY5Y cells and HEK 293 cells were seeded into each well of 96-well plates and incubated with various concentration of DMSO (5 mmol/L), methyl jasmonate (MJ, 0.5–2.5 mmol/L), *cis*-jasmone (CJ, 0.5–2.5 mmol/L), jasmonic acid (JA, 0.5–2.5 mmol/L), or cisplatin (DDP, 0.025–0.1 mmol/L) as indicated. MTT colorimetric assay indicated that methyl jasmonate, *cis*-jasmone, jasmonic acid, and cisplatin, but not DMSO, inhibited the cell proliferation of SH-SY5Y cells in a dose- and time-dependent manner(A). Moreover, the cell proliferation of HEK 293 was suppressed by cisplatin, but not by methyl jasmonate, *cis*-jasmone, or jasmonic acid (B). There was significant increase from control treated with DMSO ($^bP<0.05$). Triplicate experiments were performed with essentially identical results.

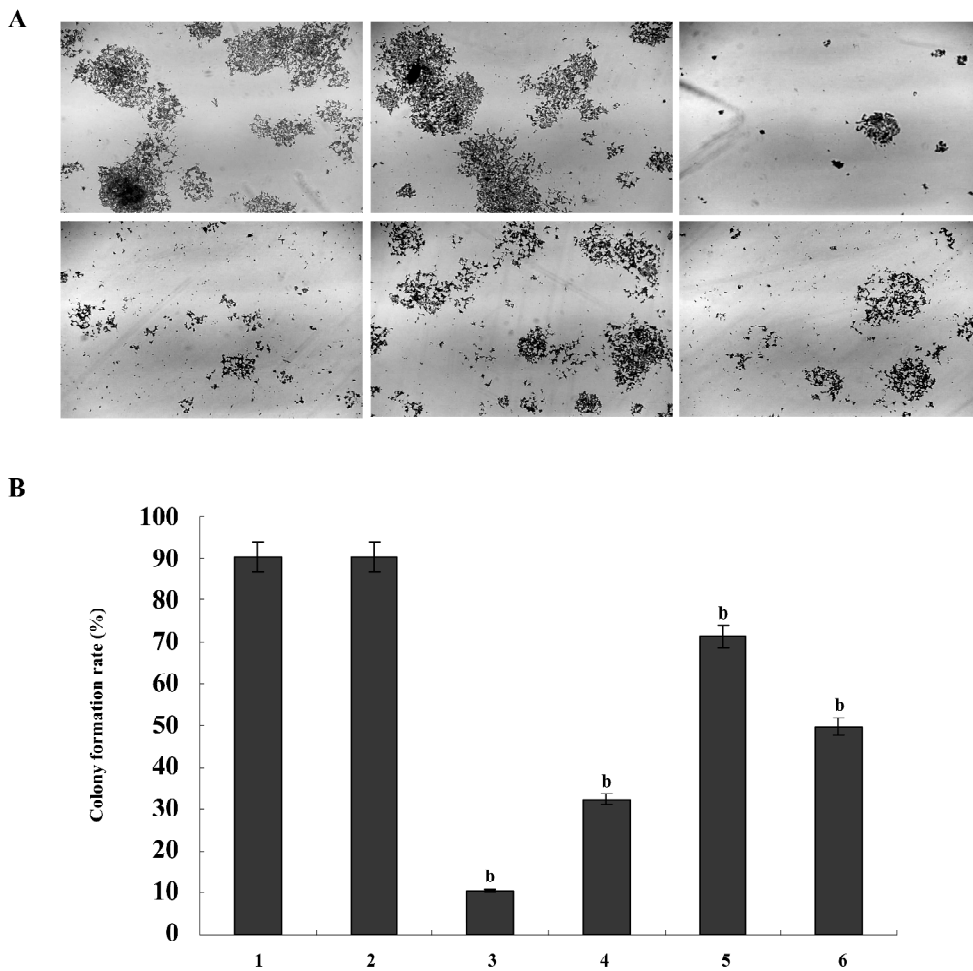


Figure 3. Natural jasmonates suppress the colony formation of SH-SY5Y cells. Confluent monolayers of SH-SY5Y cells were seeded at a density of 500/mL on 35 mm dishes. After an overnight incubation to allow cell attachment, cells were incubated with natural jasmonates, cisplatin, or DMSO as indicated. Colonies were allowed to grow for 10–14 d. (A) administration of methyl jasmonate, *cis*-jasmone, jasmonic acid, and cisplatin resulted in decreased colony formation capabilities of SH-SY5Y cells. 1: negative control; 2: 5 mmol/L DMSO control; 3: 2.5 mmol/L methyl jasmonate; 4: 2.5 mmol/L *cis*-jasmone; 5: 2.5 mmol/L Jasmonic acid; 6: 0.025 mmol/L cisplatin. (B) sensitivity of SH-SY5Y cells was found to be in the following order: methyl jasmonate>*cis*-jasmone>jasmonic acid. There was significant decrease from untreated control (^b*P*<0.05). Triplicate experiments were performed with essentially identical results.

to SH-SY5Y cells resulted in eccentric nuclei and nuclei condensation, characteristic changes of apoptosis. The Annexin V–FITC/PI staining flow cytometry was used to quantify the number of apoptotic cells induced by jasmonates. As shown in Figure 4B, natural jasmonates (2.5 mmol·L⁻¹) exerted strong apoptosis-inducing effects on neuroblastoma cells. The cellular apoptotic rates of methyl jasmonate-, *cis*-jasmone-, and jasmonic acid-treated SH-SY5Y cells were 78.9%, 37.46%, and 8.32%, respectively (Table 2), which were higher than that of untreated cells (4.29%).

Methyl jasmonate downregulates PCNA and N-myc in SH-SY5Y cells As indicated earlier, natural jasmonates suppress the cell proliferation of neuroblastoma cells, so we

hypothesized that jasmonates may modulate the expression of PCNA and N-myc, critical proteins that are involved in neuroblastoma development and progression^[13,14]. To meet this end, the SH-SY5Y cells were treated with 0.5, 1.5, and 2.5 mmol·L⁻¹ methyl jasmonate and 0.025 mmol·L⁻¹ cisplatin for 24 h. As shown in Figure 5, Western blotting demonstrated that methyl jasmonate, but not DMSO or cisplatin, decreased the expression of PCNA and N-myc in the SH-SY5Y cells in a dose-dependent manner. The administration of 0.5, 1.5, and 2.5 mmol·L⁻¹ methyl jasmonate resulted in the downregulation of PCNA and N-myc in the SH-SY5Y cells by 31.9%–64.5% and 17.1%–68.4%, respectively.

Methyl jasmonate downregulates XIAP and survivin in

Table 1. Cell cycle phases of SH-SY5Y cells treated by various drugs for 24 h. *n*=3. Mean±SD. ^b*P*<0.05, ^c*P*<0.01 vs control group.

Group	G ₀ /G ₁ (%)	S (%)	G ₂ /M (%)
Negative control	72.38±0.57	23.26±0.63	4.36±0.40
5 mmol/L DMSO	73.08±1.53	23.10±0.43	3.82±1.19
0.5 mmol/L MJ	70.76±0.74 ^b	24.15±1.72	5.09±1.49
1.5 mmol/L MJ	65.20±0.95 ^c	18.73±0.97 ^c	16.07±1.79 ^c
2.5 mmol/L MJ	59.65±2.08 ^c	19.38±1.42 ^b	20.97±0.73 ^c
0.5 mmol/L CJ	72.85±1.70	22.90±1.60	4.25±2.19
1.5 mmol/L CJ	68.24±1.01 ^c	20.55±0.71 ^c	11.21±0.31 ^c
2.5 mmol/L CJ	58.75±1.65 ^c	21.01±0.17 ^c	20.24±1.49 ^c
0.5 mmol/L JA	74.34±2.21	22.53±1.07	3.13±2.11
1.5 mmol/L JA	74.58±1.63	20.62±2.30	4.80±0.91
2.5 mmol/L JA	73.18±2.92	22.79±1.59	4.03±2.49
0.025 mmol/L DDP	85.98±2.19 ^c	9.50±0.18 ^c	4.52±2.06

MJ, methyl jasmonate; CJ, cis-jasmonate; JA, jasmonic acid; DDP, cisplatin

Table 2. Cell phenotypes of SH-SY5Y cells treated by various drugs for 24 h. *n*=3. Mean±SD. ^b*P*<0.05, ^c*P*<0.01 vs control group.

Group	Annexin V ⁻ /PI ⁻ (%)	Annexin V ⁺ /PI ⁻ (%)	Annexin V ⁺ /PI ⁺ (%)	Annexin V ⁻ /PI ⁺ (%)
Negative control	94.87±3.06	1.17±0.07	3.12±0.31	0.84±0.05
2.5 mmol/L MJ	13.54±1.35 ^c	11.38±1.12 ^c	67.52±2.01 ^c	7.56±1.47 ^c
2.5 mmol/L CJ	49.12±2.12 ^c	9.45±0.83 ^c	28.01±1.36 ^c	13.42±0.41 ^c
2.5 mmol/L JA	89.94±3.01 ^b	3.28±0.29 ^b	5.04±1.42 ^b	1.74±0.09
0.025 mmol/L DDP	48.37±2.04 ^c	5.22±1.18 ^c	19.88±0.96 ^c	26.53±1.73 ^c

SH-SY5Y cells As several published studies have demonstrated that the IAP family plays a role in oncogenesis via its effective suppression of apoptosis^[15], we hypothesized that IAP might be the targets of jasmonates in human neuroblastoma SH-SY5Y cells. To meet this end, we performed Western blotting to measure the expression of XIAP and survivin, important members of the IAP family. As shown in Figure 6, methyl jasmonate (0.5, 1.5, and 2.5 mmol·L⁻¹), but not DMSO or cisplatin, attenuated the expression of XIAP and survivin in a dose-dependent manner in SH-SY5Y cells. The administration of 0.5, 1.5, and 2.5 mmol·L⁻¹ methyl jasmonate resulted in the downregulation of XIAP and survivin in SH-SY5Y cells by 17.6%–71.4% and 21.5%–61.5%, respectively.

Discussion

Jasmonates are potent lipid regulators that mediate responses to mechanical and infectious stresses throughout the plant kingdom^[16,17]. Previous evidence has indicated that salicylic acid, another plant hormone, suppresses the proliferation of various types of cancer cells, including lymphoblastic leukemia, prostate, breast, and melanoma can-

cer cells^[18], suggesting that plant stress hormones are a promising group of plant-derived anticancer agents. A major characteristic expected of a potential anticancer agent is its ability to selectively affect cancer cells while sparing normal ones. In the current study, we demonstrated that natural jasmonates exert anticancer activity on human neuroblastoma SH-SY5Y cells. Moreover, the growth of the cultured HEK 293 cell line was not affected by jasmonates, which was consistent with previous evidence that normal cells are resistant to jasmonate-mediated cytotoxicity^[10]. These findings suggest that the identification of plant-derived molecules with known roles in plants may provide novel candidates for use in clinical oncology.

The naturally-occurring jasmonates, methyl jasmonate, *cis*-jasmonate, and jasmonic acid, have been compared *in vitro* using leukemia, lymphoma, breast, prostate, and melanoma cancer cells as targets^[5]. Their anticancer activities can also be generally summed up as methyl jasmonate>*cis*-jasmonate>jasmonic acid^[19]. Thus the methyl group appears to contribute considerably to the cytotoxic effects of MJ. In the current study, we demonstrated that natural jasmonates of dif-

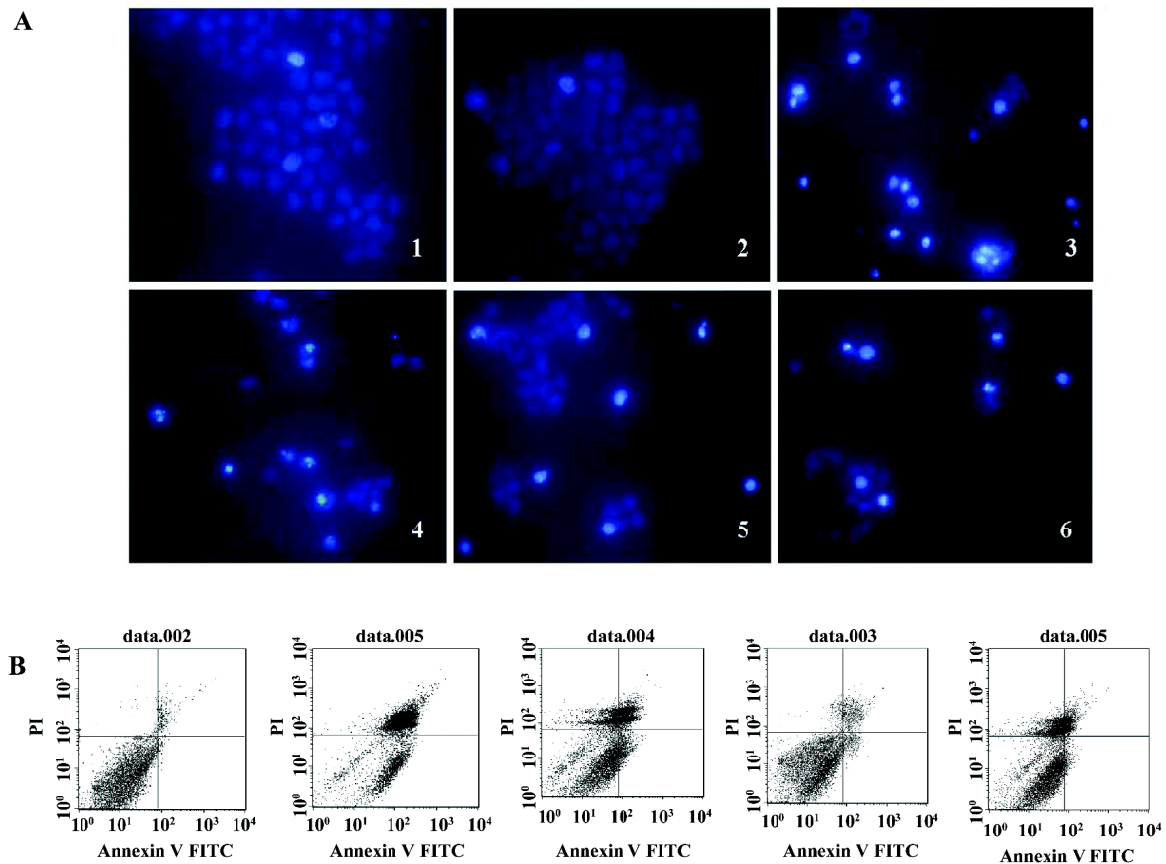


Figure 4. Natural jasmonates induce apoptosis in SH-SY5Y cells. Confluent monolayers of SH-SY5Y cells were incubated with DMSO, methyl jasmonate, *cis*-jasmone, jasmonic acid, or cisplatin for 24 h as indicated. Cells were collected for Hoechst 33258 staining and Annexin V–FITC/PI staining flow cytometry. (A) Hoechst 33258 staining indicated that the administration of methyl jasmonate, *cis*-jasmone, jasmonic acid, and cisplatin, rather than DMSO, to SH-SY5Y cells, resulted in eccentric nuclei and nuclei condensation, characteristic changes of apoptosis. 200×magnification. 1: Negative control; 2: 5 mmol/L DMSO control; 3: 2.5 mmol/L methyl jasmonate; 4: 2.5 mmol/L *cis*-jasmone; 5: 2.5 mmol/L Jasmonic acid; 6: 0.025 mmol/L cisplatin. (B) flow cytometry results indicated that natural jasmonates, methyl jasmonate, *cis*-jasmone, and jasmonic acid, induced apoptosis in cultured SH-SY5Y cells. Sensitivity of SH-SY5Y cells was found to be in the following order: methyl jasmonate>*cis*-jasmone>jasmonic acid. Triplicate experiments were performed with essentially identical results. 1: Negative control; 2: 2.5 mmol/L methyl jasmonate; 3: 2.5 mmol/L *cis*-jasmone; 4: 2.5 mmol/L jasmonic acid; 5: 0.025 mmol/L cisplatin.

ferent structures suppress the growth of human neuroblastoma SH-SY5Y cells in a dose- and time-dependent manner, and their efficiency is consistent with previous reports^[19]. Since the structures of jasmonates are different from any group of currently available anticancer agents, these findings suggest that potential future research directions should include a structure–function analysis in order to identify the actual jasmonate target molecule(s) and the discovery of new derivatives with a superior therapeutic index.

PCNA is a 36 kDa nuclear protein, and its level correlates directly with the rate of cellular proliferation^[20,21]. Oue *et al* reported that PCNA immunostaining is an appropriate index of proliferation activity in neuroblastomas. It relates well with tumor growth and other prognostic factors, and a high

PCNA expression is significantly related with a poor survival rate^[13]. Combined with the results from the colony formation assay that jasmonates inhibit the cell proliferation of SH-SY5Y cells, we hypothesized that jasmonates might influence the PCNA expression of human neuroblastoma cells. We found that methyl jasmonate downregulates the PCNA expression of cultured SH-SY5Y cells in a dose-dependent manner. We believe that PCNA is one of the target molecules of jasmonates, and the downregulation of PCNA is associated with the methyl jasmonate-induced cell proliferation inhibition of SH-SY5Y cells.

N-myc is a member of the myc family of proto-oncogenes, in which C-myc, N-myc, and L-myc have strong oncogenic potential^[22]. Approximately 25%–30% of primary untreated

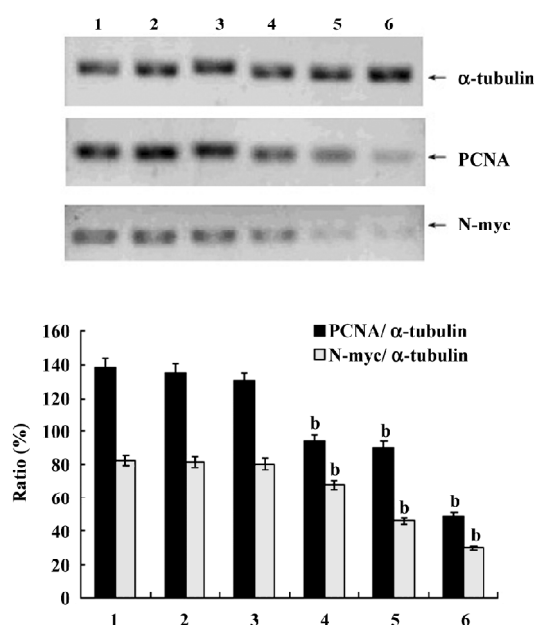


Figure 5. Methyl jasmonate downregulates the expressions of PCNA and N-myc in SH-SY5Y cells. Confluent monolayers of SH-SY5Y cells were treated with DMSO, methyl jasmonates, or cisplatin for 24 h as indicated. Western blotting demonstrated that methyl jasmonate, but not DMSO or cisplatin, decreased the expressions of PCNA and N-myc in SH-SY5Y cells in a dose-dependent manner. There was significant decrease from untreated control ($^bP<0.05$). Triplicate experiments were performed with essentially identical results. 1: Negative control; 2: 5 mmol/L DMSO control; 3: 0.025 mmol/L cisplatin; 4: 0.5 mmol/L methyl jasmonate; 5: 1.5 mmol/L methyl jasmonate; 6: 2.5 mmol/L methyl jasmonate.

neuroblastomas present amplification/overexpression of the N-myc proto-oncogene, which is associated with advanced stage disease, rapid progression, and poor prognosis^[23]. The downregulation of N-myc expression, either by antisense treatment targeted against N-myc mRNA or by retinoids, has been shown to decrease proliferation and/or induce neuronal differentiation of neuroblastoma cells^[24,25]. In the current study, we found that methyl jasmonate suppresses the N-myc expression in SH-SY5Y cells, which might be correlated with the jasmonate-mediated anticancer activity on human neuroblastoma SH-SY5Y cells.

To date, 8 human IAP have been identified, including XIAP and survivin^[26,27]. Previous evidence has indicated that in neuroblastoma cells, XIAP protects against death induced by staurosporine, thapsigargin, or serum withdrawal^[28]. In primary neuroblastomas, the survivin expression was associated with tumors of high risk and unfavorable prognosis^[29]. In the current study, we found that natural jasmonates, methyl jasmonate, *cis*-jasmone, and jasmonic acid induced apoptosis of neuroblastoma SH-SY5Y cells. The

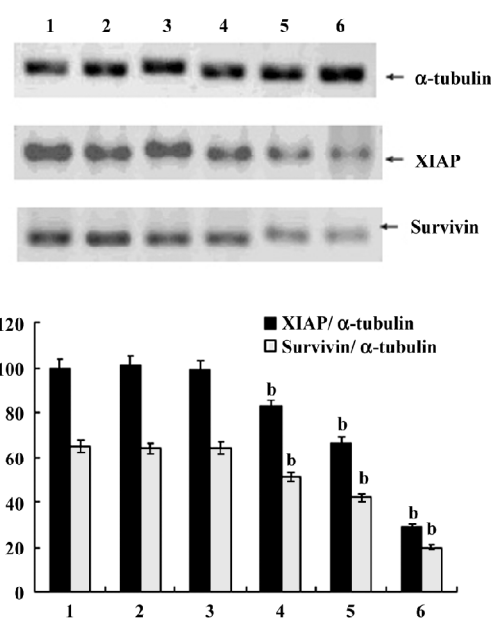


Figure 6. Methyl jasmonate downregulates the expression of XIAP and survivin in SH-SY5Y cells. Confluent monolayers of SH-SY5Y cells were stimulated with DMSO, methyl jasmonates, or cisplatin for 24 h as indicated. Western blotting demonstrated that methyl jasmonate decreased the XIAP and survivin expressions in SH-SY5Y cells in a dose-dependent manner. However, the administration of DMSO or cisplatin resulted in no changes in the XIAP and survivin levels. *b*, significant decrease from untreated control ($P<0.05$). Triplicate experiments were performed with essentially identical results. 1: Negative control; 2: 5 mmol/L DMSO control; 3: 0.025 mmol/L cisplatin; 4: 0.5 mmol/L methyl jasmonate; 5: 1.5 mmol/L methyl jasmonate; 6: 2.5 mmol/L methyl jasmonate.

expressions of XIAP and survivin were suppressed in methyl jasmonate-treated SH-SY5Y cells. We believe that jasmonates may modulate IAP to exert anticancer activity, which warrants further study.

In summary, we demonstrated that 3 kinds of natural jasmonates, methyl jasmonate, *cis*-jasmone, and jasmonic acid suppress cell proliferation and induce apoptosis in the human neuroblastoma cell line SH-SY5Y. The anticancer activities of jasmonates, are different along with their various chemical structures, which can be summed up as methyl jasmonate>*cis*-jasmone>jasmonic acid. In addition, methyl jasmonate attenuates the expression of PCNA and N-myc, and downregulates the expression of XIAP and survivin, which might be one of the mechanisms of jasmonate-mediated anticancer activity on human neuroblastoma SH-SY5Y cells.

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Author contribution

Qiang-song TONG, Fu-qing ZENG designed research; Guo-song JIANG, Li-duan ZHENG performed research; Shao-tao TANG, Ji-hua DONG contributed analytical reagents and tools; Guo-song JIANG, Jia-bin CAI, Yuan LIU analyzed data; Guo-song JIANG, Qiang-song TONG wrote the paper.

References

- 1 Schor NF. Neuroblastoma as a neurobiological disease. *J Neurooncol* 1999; 41: 159–66.
- 2 Westermann F, Schwab M. Genetic parameters of neuroblastomas. *Cancer Lett* 2002; 184: 127–47.
- 3 Nakagawara A, Rima-Nakagawara M, Scavarda NJ, Azar CG, Cantor AB, Brodeur GM. Association between high levels of expression of the TRK gene and favorable outcome in human neuroblastoma. *New Engl J Med* 1993; 328: 847–54.
- 4 Flescher E. Jasmonates in cancer therapy. *Cancer Lett* 2007; 245: 1–10.
- 5 Fingrut O, Flescher E. Plant stress hormones suppress the proliferation and induce apoptosis in human cancer cells. *Leukemia* 2002; 16: 608–16.
- 6 Kim JH, Lee SY, Oh SY, Han SI, Park HG, Yoo MA, *et al*. Methyl jasmonate induces apoptosis through induction of Bax/Bcl-XS and activation of caspase-3 via ROS production in A549 cells. *Oncol Rep* 2004; 12: 1233–8.
- 7 Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 1983; 65: 55–63.
- 8 Lebrin F, Goumans MJ, Jonker L, Carvalho RL, Valdimarsdottir G, Thorikay M, *et al*. Endoglin promotes endothelial cell proliferation and TGF-beta/ALK1 signal transduction. *EMBO J* 2004; 23: 4018–28.
- 9 Overbeeke R, Steffens-Nakken H, Vermes I, Reutelingsperger C, Haanen C. Early features of apoptosis detected by four different flow cytometry assays. *Apoptosis* 1998; 3: 115–21.
- 10 Movsesyan VA, Yakovlev AG, Dabaghyan EA, Stoica BA, Faden AI. Ceramide induces neuronal apoptosis through the caspase-9/caspase-3 pathway. *Biochem Biophys Res Commun* 2002; 299: 201–7.
- 11 Pietra G, Mortarini R, Parmiani G, Anichini A. Phases of apoptosis of melanoma cells, but not of normal melanocytes, differently affect maturation of myeloid dendritic cells. *Cancer Res* 2001; 61: 8218–26.
- 12 Ishii Y, Kiyota H, Sakai S, Honma Y. Induction of differentiation of human myeloid leukemia cells by jasmonates, plant hormones. *Leukemia* 2004; 18: 1413–9.
- 13 Oue T, Fukuzawa M, Kamata S, Okada A. Immunohistochemical analysis of proliferating cell nuclear antigen expression in human neuroblastoma. *J Pediatr Surg* 1995; 30: 528–32.
- 14 Tonelli R, Purgato S, Camerin C, Fronza R, Bologna F, Alboresi S, *et al*. Anti-gene peptide nucleic acid specifically inhibits MYCN expression in human neuroblastoma cells leading to cell growth inhibition and apoptosis. *Mol Cancer Ther* 2005; 4: 779–86.
- 15 Nachmias B, Ashhab Y, Ben-Yehuda D. The inhibitor of apoptosis protein family (IAPs): an emerging therapeutic target in cancer. *Semin Cancer Biol* 2004; 14: 231–43.
- 16 Wasternack C, Hause B. Jasmonates and octadecanoids: signals in plant stress responses and development. *Prog Nucleic Acid Res Mol Biol* 2002; 72: 165–221.
- 17 Liechti R, Farmer EE. The Jasmonate Pathway. *Science* 2002; 296: 1649–50.
- 18 Sotiriou C, Lacroix M, Lagneaux L, Berchem G, Body JJ. The aspirin metabolite salicylate inhibits breast cancer cells growth and their synthesis of the osteolytic cytokines interleukins-6 and -11. *Anticancer Res* 1999; 19: 2997–3006.
- 19 Rotem R, Heyfets A, Fingrut O, Blickstein D, Shaklai M, Flescher E. Jasmonates: novel anticancer agents acting directly and selectively on human cancer cell mitochondria. *Cancer Res* 2005; 65: 1984–93.
- 20 Bravo R, Frank R, Blundell PA, Donald-Bravo H. Cyclin/PCNA is the auxiliary protein of DNA polymerase-[delta]. *Nature* 1987; 326: 515–7.
- 21 Prelich G, Tan CK, Kostura M, Mathews MB, So AG, Downey KM, *et al*. Functional identity of proliferating cell nuclear antigen and a DNA polymerase-[delta] auxiliary protein. *Nature* 1987; 326: 517–20.
- 22 Mukherjee B, Morgenbesser SD, DePinho RA. Myc family oncoproteins function through a common pathway to transform normal cells in culture: cross-interference by Max and transacting dominant mutants. *Genes Dev* 1992; 6: 1480–92.
- 23 Brodeur GM. Neuroblastoma: biological insights into a clinical enigma. *Nat Rev Cancer* 2003; 3: 203–16.
- 24 Galderisi U, Di Bernardo G, Cipollaro M, Peluso G, Cascino A, Cotrufo R, *et al*. Differentiation and apoptosis of neuroblastoma cells: role of N-myc gene product. *J Cell Biochem* 1999; 73: 97–105.
- 25 Matsuo T, Thiele CJ. p27Kip1: a key mediator of retinoic acid induced growth arrest in the SMS-KCNR human neuroblastoma cell line. *Oncogene* 1998; 16: 3337–43.
- 26 Hunter AM, LaCasse EC, Korneluk RG. The inhibitors of apoptosis (IAPs) as cancer targets. *Apoptosis* 2007; 12: 1543–68.
- 27 Fulda S. Inhibitor of apoptosis proteins as targets for anticancer therapy. *Expert Rev Anticancer Ther* 2007; 7: 1255–64.
- 28 Yu LY, Korhonen L, Martinez R, Jokitalo E, Chen Y, Arumäe U, *et al*. Regulation of sympathetic neuron and neuroblastoma cell death by XIAP and its association with proteasomes in neural cells. *Mol Cell Neurosci* 2003; 22: 308–18.
- 29 Azuhata T, Scott D, Takamizawa S, Wen J, Davidoff A, Fukuzawa M, *et al*. The inhibitor of apoptosis protein survivin is associated with high-risk behavior of neuroblastoma. *J Pediatr Surg* 2001; 36: 1785–91.