

Ginsenoside Rh2 Induces Apoptosis *via* Activation of Caspase-1 and -3 and Up-Regulation of Bax in Human Neuroblastoma

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In human neuroblastoma SK-N-BE(2) cells undergoing apoptotic death induced by ginsenoside Rh2, a dammarane glycoside that was isolated from *Panax ginseng* C. A. Meyer, caspase-1 and caspase-3 were activated. The expression of Bax was increased in the cells treated with ginsenoside Rh2, whereas Bcl-2 expression was not altered. Treatment with caspase-1 inhibitor, Ac-YVAD-CMK, or caspase-3 inhibitor, Z-DEVD-FMK, partially inhibited ginsenoside Rh2-induced cell death but almost suppressed the cleavage of the 116 kDa PARP into a 85 kDa fragment. When the levels of p53 were examined in this process, p53 accumulated rapidly in the cells treated early with ginsenoside Rh2. These results suggest that activation of caspase-1 and -3 and the up-regulation of Bax are required in order for apoptotic death of SK-N-BE(2) cells to be induced by ginsenoside Rh2, and p53 plays an important role in the pathways to promote apoptosis.

Key words: Ginsenoside Rh2, Apoptosis, Caspase-1, Caspase-3, Bax, Neuroblastoma

INTRODUCTION

The members of Bcl-2 family play a central role in the regulation of apoptosis (Reed, 1997; Adams and Cory, 1998). Pro-apoptotic Bcl-2 members include Bax, Bad, and Bid, and anti-apoptotic members include Bcl-2 and Bcl-x_L. It appears that Bax translocates from the cytoplasm to the outer mitochondrial membrane upon induction of apoptosis, where they undergo a conformational change, oligomerize, insert into the membrane, and release cytochrome c into the cytosol (Desagher and Martiou, 2000). There, it binds to the apoptotic proteinase activating factor-1 (Apaf-1) and triggers the formation of the apoptosome that contains cytochrome c, Apaf-1, (d)ATP, and procaspase-9 (Li *et al.*, 1997; Zou *et al.*, 1999). The apoptosome-bound procaspase-9 is activated, and then, caspase-9 activates an effector caspase-3. On the other hand, the anti-apoptotic proteins Bcl-2 and Bcl-x_L work to prevent cytochrome c release from mitochondria by selective binding to Bax and preserve cell survival (Yang *et al.*, 1997; Kluck *et al.*, 1997; Kharbanda *et al.*, 1997).

Recently, it has been reported that ginsenoside Rh2, a

dammarane glycoside isolated from *Panax ginseng* C. A. Meyer, induced apoptosis in several cancer cell lines (Kim *et al.*, 1999, 2000; Park *et al.*, 1997; Fei *et al.*, 2002). Ginsenoside Rh2 induced apoptotic cell death in C6Bu-1 rat glioma without any alteration in the expression of Bcl-2, Bax, or Bcl-x_L (Kim *et al.*, 1999). In addition, overexpression of Bcl-2 in C6Bu-1 and SK-HEP-1 cells failed to prevent ginsenoside Rh2-induced cell death (Kim *et al.*, 1999; Park *et al.*, 1997). It was observed that differential modulation of protein kinase C (PKC) subtypes during the apoptotic death was induced by ginsenoside Rh2 in SK-N-BE(2) cells, but the lack of significant changes in PKC subtypes in C6Bu-1 cells suggested that ginsenoside Rh2 might induce apoptosis via different pathways that interact with or without PKC in different cell types (Kim *et al.*, 2000). However, it has not been described that certain mechanisms mediate ginsenoside Rh2-induced apoptosis in SK-N-BE(2) cells. In the present study, we aimed to understand the mechanisms of apoptotic cell death induced by ginsenoside Rh2 in SK-N-BE(2) cells. The data demonstrated that ginsenoside Rh2 activated caspase-1 and -3 to induce the apoptotic death of SK-N-BE(2) cells and ginsenoside Rh2 up-regulated Bax expression. However, ginsenoside Rh2-induced cell death was partially inhibited by caspase inhibitors, and p53 tumor suppressor protein is involved in this apoptosis.

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MATERIALS AND METHODS

Materials

Ginsenoside Rh2 from *Panax ginseng* was prepared as previously described (Kim *et al.*, 1999; 2000) and its purity was over 99% according to the mass spectrum and ¹³C-NMR spectroscopy. Fetal bovine serum, trypsin and Dulbecco's Modified Eagle's Medium (DMEM) and other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). Antibodies to caspase-1 (sc-622 and sc-1780), β -actin (sc-1615), and Bax (sc-493-G) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Antibodies to caspase-3 (C31720), Bcl-2 (B46620), and p53 (P21020) were purchased from Transduction Laboratories (Lexington, KY). The antibody to PARP was purchased from Boehringer Mannheim (Mannheim, Germany). Ac-Tyr-Val-Ala-Asp-chloromethylketone (Ac-YVAD-CMK) and benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethylketone (Z-DEVD-FMK) were purchased from Calbiochem (La Jolla, CA).

Cell culture

Human neuroblastoma SK-N-BE(2) cells were cultured in DMEM medium that was supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/mL of penicillin and 100 μ g/mL of streptomycin at 37°C in 5% CO₂ humidified atmosphere. The cell line was subcultured two times per week.

Cell viability

The cells (2×10^5 cells/well/mL) were plated on 24 well plates, maintained for 24 h and treated in fresh medium with ginsenoside Rh2 in a final concentration of 0.5% ethanol for the appropriate time. As previously described (Kim *et al.*, 1999, 2000), the concentration of ethanol had no effect on cell growth and morphology. Caspase inhibitors were dissolved in 100% DMSO, kept at 4°C as a stock solution, and diluted immediately with fresh DMEM to 0.05% DMSO in culture. The inhibitors were pretreated for 1 h before being treated with ginsenoside Rh2. Cell viability was assayed with the sulforhodamine B (SRB) method (Skehan *et al.*, 1990).

Flow cytometry

Adherent cells were trypsinized and combined with non-adherent cells. $1-2 \times 10^6$ cells were collected by centrifugation at 400 g for 5 min, washed with phosphate-buffered saline (PBS), and resuspended in 0.2 mL of PBS. Then 0.8 mL of absolute ethanol was added sequentially to the cell suspension, and the mixture was incubated overnight at 4°C in order to fix the cells. Fixed cells were pelleted, washed with PBS, and stained in the dark for 1 h with 50 μ g/mL of propidium iodide in 0.5 mL of PBS containing 40

μ g/mL of RNase A. The stained cells were analyzed by FACSort (Becton-Dickinson).

Electron microscopy

Cells grown on Lab-Tek chambered slides were prefixed in cacodylate-buffered glutaraldehyde (2%), post-fixed in 1% osmium tetroxide, dehydrated in graded series of alcohol, and embedded in Epon (PolyBed 812). Sections were stained with uranyl acetate and lead citrate and were examined with a Zeiss EM-900 electron microscope.

Immunoblotting

The cells ($2 \times 10^6-1 \times 10^7$ cells) were washed once with ice-cold PBS, sonicated (10 sec, 2 cycles) at 4°C in 200 μ L of extraction buffer (10 mM Hepes, pH 7.4, 10 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, 50 mM NaF, 1 mM phenylmethylsulphonyl fluoride, 10 μ g/mL leupeptin, 20 μ g/mL aprotinin), and incubated in ice for 10 min. Cellular lysate was recovered after centrifugation at 10,000 \times g for 10 min at 4°C. The protein was determined by a Bio-Rad protein assay kit with bovine serum albumin as the standard. Equal amounts of protein (15~30 mg) were subjected to 8~12% SDS-PAGE and were transferred to nitrocellulose (Hybond-ECL, Amersham). The individual target protein was immunoreacted with the appropriate dilution of its primary antibodies (caspase-1, caspase-3, 1: 200 dilution; Bcl-2, Bax, 1:500 dilution; PARP, 1:2,000 dilution; p53, 1:400 dilution; actin, 1:400 dilution) at room temperature for 1 h or 2 h, and the protein was incubated with an appropriate dilution of horseradish peroxidase conjugated anti-rabbit IgG antibody (1:2,000 dilution, Sigma Chemical Co.), anti-mouse IgG antibody (1:670 dilution, Amersham), or anti-goat IgG antibody (1:1,000 dilution, Sigma Chemical Co.) as a secondary antibody. Enhanced Chemiluminescence (ECL, Amersham) was used to reveal antibody binding.

Statistical analysis

Data are expressed as the mean \pm SD of three independent experiments performed in triplicate. Significant differences between means were assessed by the student's *t*-test.

RESULTS

Induction of apoptosis

In the previous report (Kim *et al.*, 2000), apoptotic cell death in SK-N-BE(2) cells was induced by ginsenoside Rh2 in a dose-dependent manner, which was assessed by characteristic morphological changes, DNA fragmentation and *in situ* strand breaks. In this study, apoptotic cell death was confirmed by the flow cytometric method and

the ultra-structural analysis. The treatment of SK-N-BE(2) cells with 50 μ M ginsenoside Rh2 for 0, 3, 8, and 24 h increased the proportion of cells with sub-G1 DNA content to 2.61, 8.58, 32.04, and 81.74% in a time-dependent manner (Fig. 1). The electron microscopic observation revealed typical apoptotic features, including shrinkage of cellular and nuclear membranes, condensed heterochromatin around the nuclear periphery, and cytoplasmic vacuolation in the cells treated with 50 μ M ginsenoside Rh2 for 24 h (Fig. 2).

Activation of Caspase-1 and -3 by ginsenoside Rh2

Caspase-1 and -3 are cysteine proteases that exist as an inactive zymogen in cells and are activated by sequential proteolytic events that cleave their precursor at aspartic acid residues to generate an active heterodimer. In the cells treated with 50 μ M ginsenoside Rh2 for 0, 3, 6, 16, and 24 h, ginsenoside Rh2 induced the proteolytic cleavage of procaspase-1 after 3 h of treatment (Fig. 3A). Procaspase-3 decreased gradually after exposure to ginsenoside Rh2, as well as to a well known caspase-3 substrate, 116 kDa PARP cleaved to a 85 kDa of fragment in a time-dependent manner (Fig. 3B). These results indicated that the proteolysis of procaspase-3 to an active form resulted in cleavage of PARP in the cells undergoing apoptosis induced by ginsenoside Rh2.

Induction of Bax by ginsenoside Rh2

As shown in Fig. 4, the level of Bax increased in a time-dependent manner after 6 h of treatment with 50 μ M ginsenoside Rh2. On the other hand, the expression of

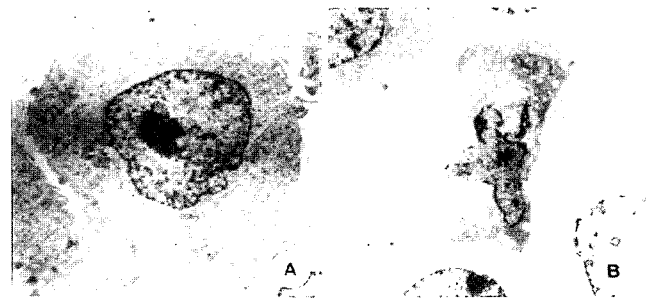


Fig. 2. Ultrastructural analysis of SK-N-BE(2) cells treated with or without 50 μ M ginsenoside Rh2 for 24 h. Samples were fixed and prepared for electron microscopy. A, untreated cell (x 4000); B, ginsenoside Rh2-treated cell (x 4000).

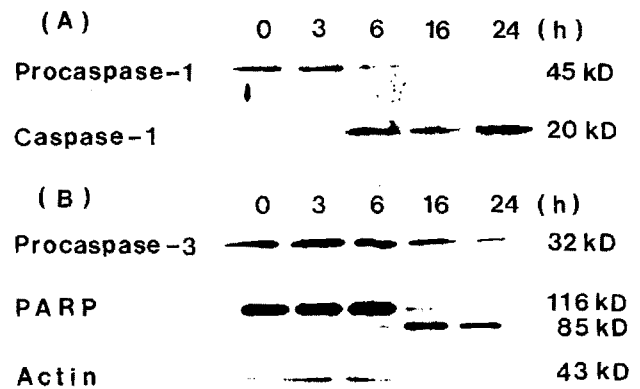


Fig. 3. Activation of caspase-1 (A) and caspase-3 (B) during ginsenoside Rh2-induced apoptosis in SK-N-BE(2) cells. The cells were treated with 50 μ M ginsenoside Rh2 for 0, 3, 6, 16, and 24 h. Cell lysates were prepared and were subjected to 8-12% SDS-PAGE electrophoresis. Western blotting was performed as described in Materials and Methods.

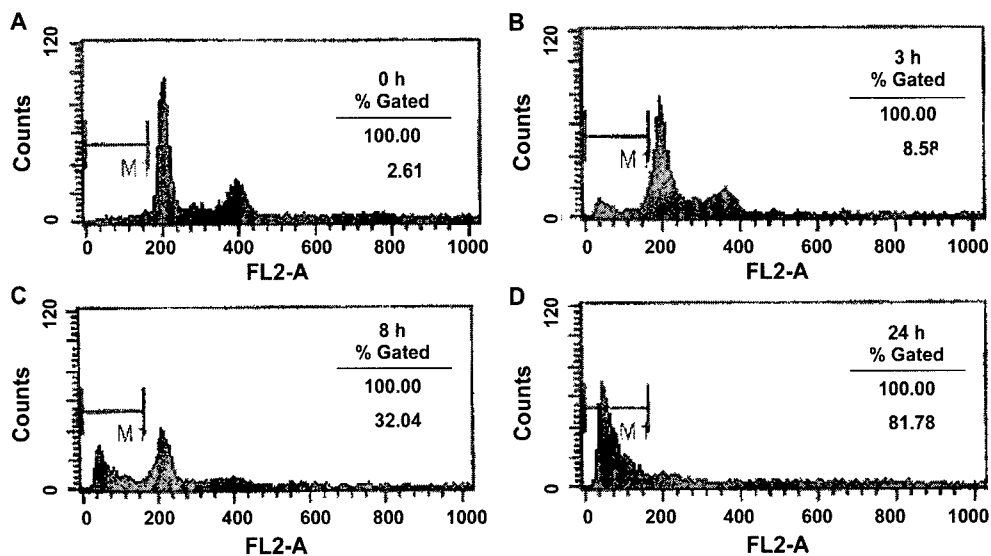


Fig. 1. Induction of apoptosis by ginsenoside Rh2 in SK-N-BE(2) cells. The cells were treated with 50 μ M ginsenoside Rh2 for 0 h (A), 3 h (B), 8 h (C), and 24 h (D) and were fixed with ethanol followed by staining with propidium iodide. The population of apoptotic cells was determined by flow cytometry. Sub G1 phase cells (M) are represented as a horizontal bar.

Bcl-2 was not altered during the process of ginsenoside Rh2-induced apoptosis.

Effects of caspase inhibitors on apoptosis induced by ginsenoside Rh2

The activation of caspase-3-like proteases is a critical step in the Bax-mediated apoptotic pathway (Kitanaka *et al.*, 1997; Xiang *et al.*, 1996; Jürgensmeier *et al.*, 1998; Finucane *et al.*, 1999; Pastorino *et al.*, 1998). It was demonstrated that Z-VAD-FMK, a general caspase inhibitor, suppressed activation of caspase-3-like protease and

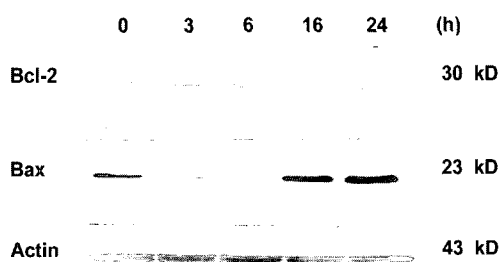


Fig. 4. The expression of Bax and Bcl-2 in ginsenoside Rh2-treated SK-N-BE(2) cells. The cells were treated with 50 μM ginsenoside Rh2 for 0, 3, 6, 16, and 24 h followed by Western blotting as described in Materials and Methods.

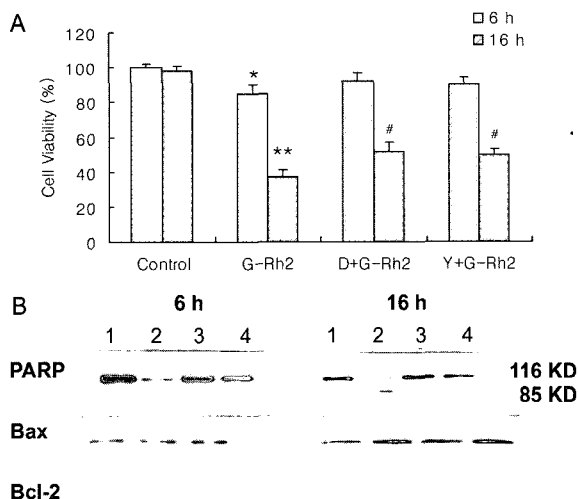


Fig. 5. Effects of caspase inhibitors on cell viability (A), PARP cleavage, Bcl-2 and Bax expression (B) in SK-N-BE(2) cells treated with ginsenoside Rh2. The cells (2×10^5 /mL/well) were pretreated with or without 50 μM of each inhibitor (D, Z-DEVD-FMK; Y, Ac-YVAD-CMK) for 1 h and treated with 50 μM ginsenoside Rh2 for 6 h or 16 h. The viability was determined using the SRB method. The data are shown as the mean \pm SD of three independent experiments performed in triplicate. *: significant at $p < 0.05$ or **: significant at $p < 0.01$, respectively, compared to the control. #: significant at $p < 0.05$, compared to the G-Rh2 treatment. For Western blot analysis, cell lysates were prepared and subjected to 8-10% SDS-PAGE electrophoresis. Lane 1, untreated control; Lane 2, ginsenoside Rh2; Lane 3, Z-DEVD-FMK+ginsenoside Rh2; Lane 4, Ac-YVAD-CMK+ginsenoside Rh2.

apoptotic cell death that was induced by Bax (Kitanaka *et al.*, 1997). However, caspase inhibitors failed to prevent Bax-induced cell death but inhibited the activation of caspases (Xiang *et al.*, 1996; Jürgensmeier *et al.*, 1998). We, therefore, examined the effects of caspase inhibitors on cell death and activation of caspase-1 and -3 induced by ginsenoside Rh2. As shown in Fig. 5A, the treatment of cells with ginsenoside Rh2 for 6 and 16 h resulted in $85 \pm 5\%$ and $37 \pm 4\%$ of cell viability, respectively. The cells that were pretreated with Z-DEVD-FMK caspase-3-specific inhibitor for 1 h before treatment with ginsenoside Rh2 for 6 and 16 h presented cell viabilities of $92 \pm 4\%$ and $52 \pm 5\%$, respectively. In the case of Ac-YVAD-CMK caspase-1-specific inhibitor pretreatment under the same conditions, cell viability was $90 \pm 6\%$ and $50 \pm 3\%$ at 6 and 16 h, respectively. These results showed that the inhibiting effect by both inhibitors was little but to a level that is statistically significant. On the other hand, the caspase inhibitors almost suppressed the cleavage of 116 kDa PARP into a 85 kDa fragment in the cells treated with ginsenoside Rh2 for 6, or 16 h, but they did not alter the level of Bax and Bcl-2 in the cell treated with ginsenoside Rh2 (Fig. 5B).

Effects of ginsenoside Rh2 on level of p53

It has been shown in several cell types that the wild type p53 is activated by a variety of stimuli such as irradiation and anticancer drugs, which promotes apoptosis (Vogelstein *et al.*, 2000). Therefore, the levels of p53 were detected in the cells treated with 50 μM ginsenoside Rh2 for 0, 3, 6, 16, or 24 h. The level of p53 increased within the first three hours of treatment (Fig. 6).

DISCUSSION

This study illustrated that ginsenoside Rh2-induced apoptotic cell death was accompanied by the activation of caspase-1- and -3 and the up-regulation of Bax. However, caspase inhibitors partially inhibited ginsenoside Rh2-induced apoptosis but suppressed PARP cleavage in the cells undergoing apoptosis. The accumulation of p53 was

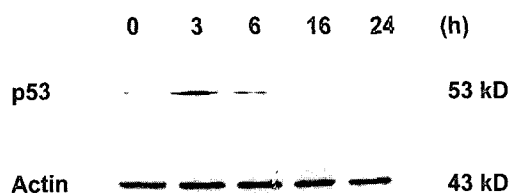


Fig. 6. Expression of the p53 tumor suppressor protein in SK-N-BE(2) cells undergoing apoptosis induced by ginsenoside Rh2. The cells were treated with 50 μM ginsenoside Rh2 for 0, 3, 6, 16, and 24 h followed by Western blotting as described in Materials and Methods.

evident in early stages of treatment of SK-N-BE(2) cells with ginsenoside Rh2.

It has been shown that the activation of caspase-3-like protease is essential for Bax-induced apoptosis (Kitanaka *et al.*, 1997; Xiang *et al.*, 1996; Jürgensmeier *et al.*, 1998; Finucane *et al.*, 1999). However, Bax-induced cell death was not inhibited by the treatment with Z-VAD-FMK or Z-DEVD-FMK (Xiang *et al.*, 1996; Jürgensmeier *et al.*, 1998; Pastorino *et al.*, 1998). These studies showed that Bax can possibly mediate apoptosis independently of caspases, which could promote the mitochondrial permeability or could puncture the mitochondrial outer membrane. Although it is controversial whether Bax mediates cell death by the regulation of caspases or through the induction of membrane permeability that is insensitive to caspase inhibitors, it indicates that Bax acts upstream of caspase-3-like proteases and regulates their activity. Therefore, our results showed that the caspase-3 inhibitor partially inhibited ginsenoside Rh2-induced cell death but had no effect on the up-regulated Bax expression. This suggests that Bax regulates the activation of caspase-3 in the cells treated with ginsenoside Rh2, and ginsenoside Rh2 mediates another pathway, such as caspase-insensitive or -independent pathway, to induce apoptosis. Procaspase-9 is an initiator caspase of the apoptosome and allows the activation of down-stream caspases such as caspase-3. Therefore, caspase-9 possibly acts upstream of caspase-3 in other to execute apoptosis in ginsenoside Rh2-treated cells.

It was reported that ginsenoside Rh2 induced apoptosis independently of Bax, Bcl-2 or Bcl-_{XL} in C6Bu-1 cells (Kim *et al.*, 1999). However, this study showed that ginsenoside Rh2 promoted death of SK-N-BE(2) cells by increasing the amount of free Bax or the Bax homodimer. These results indicated that ginsenoside Rh2 might mediate diverse pathways from cell line to cell line, finally leading to apoptosis.

It has been reported that activation of caspase-1 cleaves procaspase-3 to generate active caspase-3, where the cleavage of PARP *in vitro* (Tewari *et al.*, 1995) and Ac-YVAD-CHO or Ac-YVAD-CMK inhibited apoptosis that was induced by Fas (Enari *et al.*, 1996), ceramide (Smyth *et al.*, 1996), a Ca²⁺ ionophore, or hypoxia (Shimizu *et al.*, 1996). This suggests that the activation of caspase-1-like proteases results in the activation of caspase-3-like proteases. In this study, Ac-YVAD-CMK caspase-1 inhibitor suppressed PARP cleavage and protected partly from cell death as the Z-DEVD-FMK caspase-3 inhibitor did, and this suggests that caspase-1 can catalyze procaspase-3 into an active caspase-3 in ginsenoside Rh2-induced apoptosis of SK-N-BE(2) cells. Although the role of caspase-1 in ginsenoside Rh2-induced apoptosis is unclear in this study, these results could support the operation of a

possible caspase cascade to transducer death signals in apoptosis of SK-N-BE(2) cells.

p53 can also induce apoptosis in a transcription-dependent manner through the induction of genes such as Bax (Miyashita and Reed, 1995). It has been described that caspase activation by p53 occurs through the release of cytochrome c from the mitochondria, which can be triggered by Bax activation (Schuler *et al.*, 2000; Schuler and Green, 2001). In this study, the level of p53 accumulated at an early stage in the process of apoptosis induced by ginsenoside Rh2, suggesting that p53 may be involved in the up-regulation of Bax expression and/or another pathway including caspase activation.

Taken together, this study suggests that the activation of caspase-1 and -3 and Bax up-regulation are required for ginsenoside Rh2-induced apoptosis in SK-N-BE(2) cells, and p53 is involved in the mechanism of cell death.

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