Department of Neurosurgery[1], The First Affiliated Hospital of Nanjing Medical University, Nanjing City; Department of Oncology[2], Jinling Hospital, Nanjing University, Nanjing, China

SP600125, a JNK inhibitor, suppresses growth of JNK-inactive glioblastoma cells through cell-cycle G2/M phase arrest

J.Y. Li[1], J. Y. Huang[2], B. Xing[1], K. W. Ren[1], M. Li[1], D. Wei[1], P. Y. Gu[1], G. Chen[1], B. Gu[1], G. F. Zhang[1], W. X. Hu[1]

Received December 16, 2011, accepted February 10, 2012

Dr. Weixing Hu, The First Affiliated Hospital of Nanjing Medical University, 300 Guangzhou Road, Nanjing City, 210029, China
hwx66@126.com

Pharmazie 67: 942–946 (2012) *doi: 10.1691/ph.2012.1849*

SP600125 is a well studied inhibitor of c-Jun N-terminal kinase (JNK). Its direct biochemical effects on JNK-inactive tumor cells are usually ignored. In this study, we investigated the effects of SP600125 on JNK-inactive U251 human glioblastoma cells. Our results demonstrate that, 20 μM or more SP600125 can induce significant cell growth inhibition and cell-cycle G2/M phase arrest in U251 cells. Interestingly, we also found that SP600125 can stop the duplicated chromosomes from separating into two cells and the karyokinesis progression. Our study opened up a new perspective for further studies involved in JNK inhibitors or anti-glioma therapy.

1. Introduction

The c-Jun N-terminal kinase (JNK) is a family of serine/threonine protein kinases activated by a range of stress stimuli, able to phosphorylate the N-terminal transactivation domain of the c-Jun transcription factor and regulate transcription through its effects on several transcription factors (Jeong et al. 2010; Yi et al. 2010). JNK-dependent signaling events were considered to be very important in normal development or diseases. Inhibitors have been used increasingly to explore the biological functions of JNK in mammalian systems without the need for JNK gene knockout (Bogoyevitch et al. 2010).

SP600125 is an anthrapyrazolone ATP-competitive and highly selective JNK inhibitor. It was widely used to dissect signalling mechanisms and JNK-dependent cell events. Most studies related to SP600125 were concerned with its JNK inhibition activity; hardly any reports had ever mentioned its direct biochemical effects on JNK inactive events. In this study, we investigated the effects of SP600125 on U251 human glioblastoma cells. Our results indicated that SP600125 can also be used to inhibit glioblastoma cell growth. This opened up a new perspective in studies concerning on JNK inhibitors and glioma-therapy.

2. Investigations and results

2.1. SP600125 suppressed U251 cell growth

Cells were treated with SP600125 at concentration of 1, 5, 10, 20, 50 or 100 μM for 48 h. A CCK-8 kit was used to detect cell viability as mentioned in Materials and Methods section. The optical density (OD) values represented the cell viabilities. Cells treated with DMSO (final concentration was 0.1%) were used as the control. As shown in Fig. 1, SP600125 suppressed U251 cell growth in a concentration-dependent manner ($^*P < 0.01$).

2.2. SP600125 induced cell-cycle G2/M phase arrest and attenuated CDC2 activation

Cells were treated with 20, 50 or 100 μM SP600125 for 96 h and cell cycle progression was evaluated after propidium iodide staining by fluorescence-activated cell-sorting analysis. Cells treated with DMSO (final concentration was 0.1%) were used as vehicle control. As the results shown in Fig. 2, the proportion of cells in the G2/M phase increased in a concentration-dependent manner from 13.47% (vehicle control) to 75.74% (20 μM) to 85.17% (50 μM) to 85.76% (100 μM), with a corresponding decrease in cells in the G1 and S phases.

The activation of CDC2 was detected by immunoblotting. Antibody against phosphorylaed-CDC2 (Thr161) was used. Cells were treated with 50 μM SP600125 for 30 min, 1 h, 2 h, 4 h, 48 h or 96 h. Cells treated with DMSO (final concentration was 0.1%) were used as vehicle control and exhibited a high level of CDC2 activation. SP600125 attenuated CDC2 activation in a time-dependent manner (Fig. 3A).

As shown in Fig. 3B, almost no phosphorylated-JNK was detected in U251 cells. JNK is inactive in U251 cells. Anisomycin treated cell samples were used as a positive control, and the level of unphosphorylated JNK was detected as a loading control.

2.3. SP600125 caused unique nuclear vacuolization in U251 cells

U251 cells were treated with 5, 20 or 50 μM SP600125 for 48 h. DMSO treated cells were used as vehicle control. Then, Hoechst 33342 nuclear staining was performed. Fig. 4, the vacuolated nuclei are indicated by black arrows. 10 random visual fields (200×) were selected in each sample and the average number of vacuolated nuclei in these fields was calculated. After SP600125

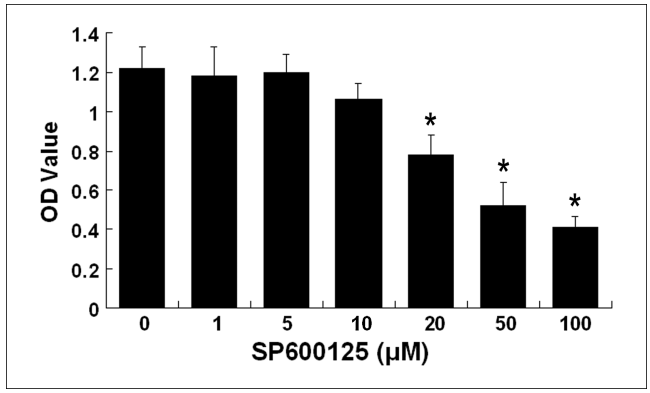

Fig. 1: SP600125 suppressed U251 cell growth. Cells were treated with SP600125 at concentration of 1, 5, 10, 20, 50 or 100 μM for 48 h. The optical density (OD) values represented the cell viabilities. SP600125 inhibits U251 cell growth in a concentration-dependent manner (*$P < 0.01$)

treatment, the number of cells with vacuolated nucleus increased in a concentration-dependent manner from 0 per field (vehicle control) to 4 ± 1 per field (5 μM) to 48 ± 8 per field (20 μM) and to 63 ± 4 per field (50 μM).

3. Discussion

JNK-dependent signaling events were considered to be very important in normal development and in progression of diseases. Inhibitors have been used increasingly to explore the role of JNK in various physiological processes and treatment of diseases (Bogoyevitch et al. 2010). Among them, SP600125 is the best studied and widely used. JNK inhibition by SP600125 was observed to be reversible, ATP-competitive and highly selective (Bennett et al. 2001; Duan and Wong 2006). SP600125 was not only used to dissect signalling mechanisms of JNK pathway *in vitro*, but also found to have efficacy in the treatment of viral infection and various insults including ischemia and ischemia/reperfusion damage (Bogoyevitch and Arthur 2008). In some cases, SP600125 was also reported to modulate immune cell responses, and thus provide beneficial effects (Hamza et al. 2004). In the area of tumor research, SP600125 was usually used to inhibit the high activity of JNK in tumor cells and then consequently suppress tumor cell growth and induce cell apoptosis (Yi et al. 2010).

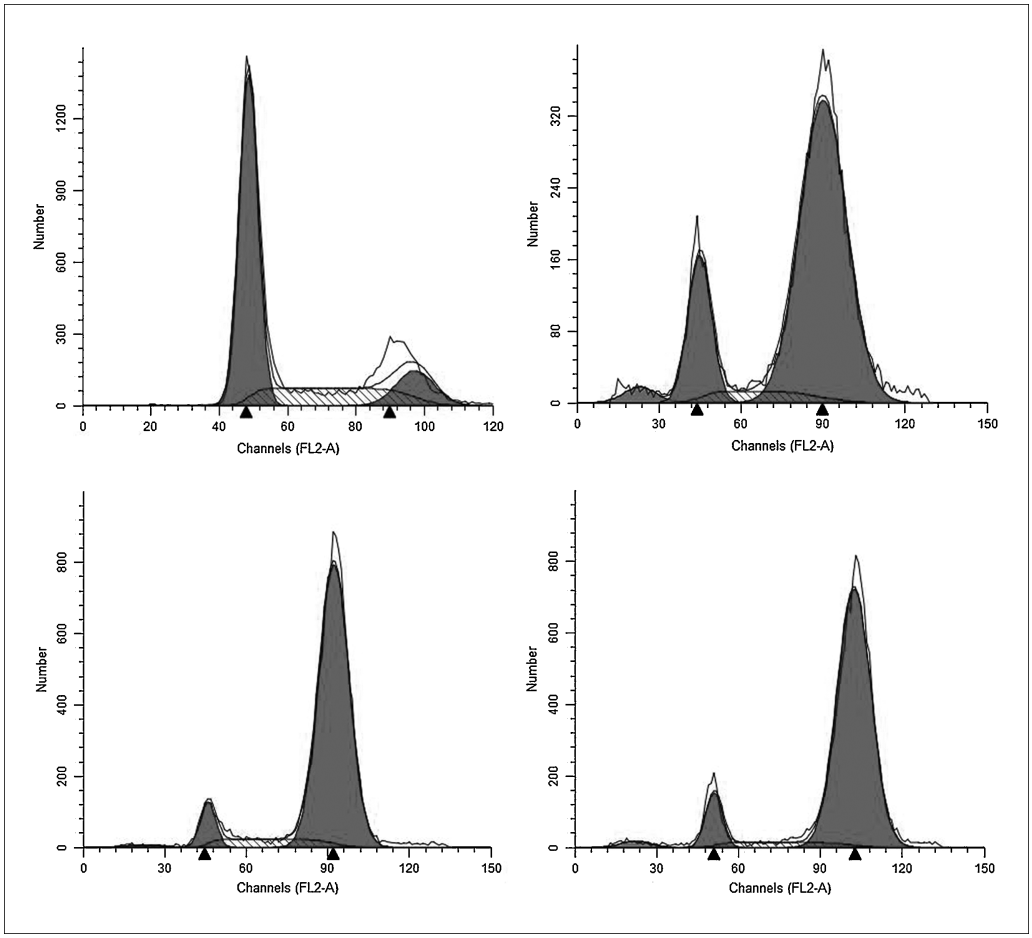

Fig. 2: SP600125 induced cell-cycle G2/M phase arrest. (A) Flow cytometry for vehicle control cells. (B–D) Cells treated with 20, 50 or 100 μM SP600125 for 96 h were evaluated after propidium iodide staining by fluorescence-activated cell-sorting analysis

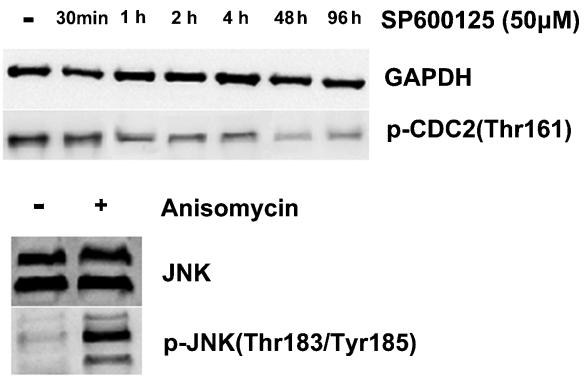

Fig. 3: A) SP600125 attenuated CDC2 activation in U251 cells. Cells were treated with 50 μM SP600125 for 30 min, 1 h, 2 h, 4 h, 48 h or 96 h. Cells treated with DMSO (final concentration was 0.1%) were used as vehicle control and exhibited a high level of CDC2 activation. SP600125 attenuated CDC2 activation in a time-dependent manner starting at 30 min after treatment. (B) Phosphorylation of JNK was detected by western blotting using antibody against phosphorylated JNK (Thr183/Tyr185). JNK was inactive in U251 cells. Anisomycin treated cell samples were used as positive control, and the level of unphosphorylated JNK was detected as loading control

However, almost all studies relate to SP600125 were focused on its JNK inhibition activity; hardly any reports mentioned its direct biochemical effects on JNK-independent events. To investigate the activities of SP600125 in JNK-inactive events, especially in JNK-inactive tumor cells, we used U251 human glioblastoma cell line as a JNK-inactive model (as shown in Fig. 3B, JNK is inactive in U251 cells). SP600125 was reported to inhibit JNK activity with an IC50 of 5 to 10 μM (Bennett et al. 2001). Our results show that SP600125 also suppressed U251 cell growth at a concentration of 20 μM or more without inhibiting JNK activity (Fig. 1). The growth inhibition activity of SP600125 may due to its effect on cell cycle progression. The first clue is the result of cell-cycle analysis (Fig. 2). After treatment of SP600125, the proportion of U251 cells in G2/M phase significantly increased in a concentration-dependent manner from 13.47% (vehicle control) to 75.74% (20 μM) to 85.17% (50 μM) and to 85.76% (100 μM), with a corresponding decrease in cells in the G1 and S phases. Then, we found the activated (phosphorylated) CDC2 in U251 cells can be promptly inactivated (dephosphorylated) by SP600125 (Fig. 3A). Phosphorylation of CDC2 at site Thr161 is one of the key steps promoting cell cycle progression across G2-phase entry into M-phase (Yoon et al. 2011). Moreover, in SP600125 treated U251 cells, unique nuclear vacuolization was detected by Hoechst 33342 nuclear staining, which indicated that SP600125 can stop the duplicated chromosomes from separating into two cells and cause karyokinesis arrest (Fig. 4).

In the present study, we described a scarce phenomenon of nuclear vacuolization in SP600125 treated U251 cells. As shown in Fig. 4 (indicated by black arrows), some nuclei have a vacuole located in the center. The vacuole separated the oval nucleus and incompletely divided chromosomes in the cell. In other words, the cell with nuclear vacuolization failed to separate duplicated chromosomes into two cells and was arrested in the process of cell division. By considering the obvious G2/M phase arrest (Fig. 2), we propose this phenomenon of nuclear vacuolization in SP600125 treated U251 cells is the representation of karyokinesis arrest.

Although SP600125 exhibited an inhibitory effect on glioblastoma cell growth and a potential ability for glioma chemotherapy, its administration in high dosage may cause serious detrimental effects including liver damage, heart failure and immune response disorder, and this may limit the application of SP600125 in treatment of JNK-inactive tumors. More investigation is needed to reveal the effects of SP600125 on other JNK-inactive tumor cells. Otherwise, there may be some other JNK inhibitors or other kinases inhibitors that have similar anti-tumor efficacy but with lower detrimental effects.

Collectively, our data clearly demonstrated that SP600125 suppresses U251 human glioblastoma cell growth and induces cell-cycle G2/M phase arrest when its concentration reaches 20 μM or more. Moreover, we described a phenomenon of nuclear vacuolization in SP600125 treated U251 cells which represented the karyokinesis arrest. Not only SP600125 can inhibit JNK activity in cells at a lower concentration, but also inhibit glioblastoma cell growth at a higher concentration (no less than 20 μM) without inhibiting JNK activity. Further investigation is needed to reveal the effects of SP600125 on other tumor cell lines.

4. Experimental

4.1. Agents and treatments

SP600125 and anisomycin were purchased from Sigma-Aldrich (St. Louis, MO, USA). In all assays, SP600125 and anisomycin were dissolved in DMSO and subsequently diluted in serum-supplemented medium immediately before use. DMSO concentration never exceeded 0.1% (v/v).

4.2. Cell culture

U251 human glioblastoma cell line was a gift from Professor Kun Yao (Department of Microbiology, Nanjing Medical University, Nanjing, China). Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum, glutamine, nonessential amino acids, and 1% penicillin/streptomycin (complete medium). Cells were grown at 37 °C in a humidified atmosphere of 95% air and 5% CO2.

4.3. Cell viability assay

Cell viability assay was performed using the cell counting kit-8 (CCK-8) kit (Dojindo Laboratories, Kumamoto, Kyushu, Japan). According to the manufacturer's instructions, cells were plated in a 96-well plate at a density of 5000 cells/well in 200ul of culture medium together with SP600125. The final dimethyl sulfoxide (DMSO) vehicle concentration did not exceed 0.1% (v/v). Then, the cells were cultured in a humidified incubator containing 5% CO2. After 48 h, 20ul CCK-8 solution was added to each well and incubated for one hour. The absorbance was measured at 450 nm using an enzyme-linked immunosorbent assay plate reader (Bio-Rad Laboratories, Inc., Berkeley, CA, USA).

4.4. Cell-cycle analysis by flow cytometry

For flow cytometric analysis, cells were plated in 6 cm culture dishes. After adherence for 6 h, cells were exposed to 0, 20, 50 or 100 μM SP600125 for 96 h at 37 °C. Cells were subsequently collected by trypsinization, centrifuged (3500 rpm for 5 min), and washed twice with PBS. After being fixed by 1 ml ethanol (70%), cells were pelleted by centrifugation (3500 rpm for 5 min), rinsed twice with PBS, and incubated with propidium iodide. A total of 10,000 nuclei were analyzed in a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA).

4.5. Western blotting

Cells were washed and lysed. Cell lysates were adjusted to the same total protein content, then boiled for 8 min in SDS loading buffer and separated by SDS-PAGE before being transferred to PVDF membranes. Membranes were blocked for 1.5 h at room temperature in TBST (0.1% Tween-20 + TBS) containing 5% (w/v) non-fat dry milk. Membranes were probed overnight at 4 °C with primary antibodies and followed by incubation for 1 h at room temperature with 1:5000 horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology, Beverly, MA, USA) in blocking solution. Then, membranes were incubated in Super Signal West Pico chemiluminescent substrate (Pierce, Chicago, USA). Developing was performed and the images were captured. For all western blots, three images were analyzed. Antibodies against GAPDH, phosphorylaed-CDC2 (Thr161), JNK

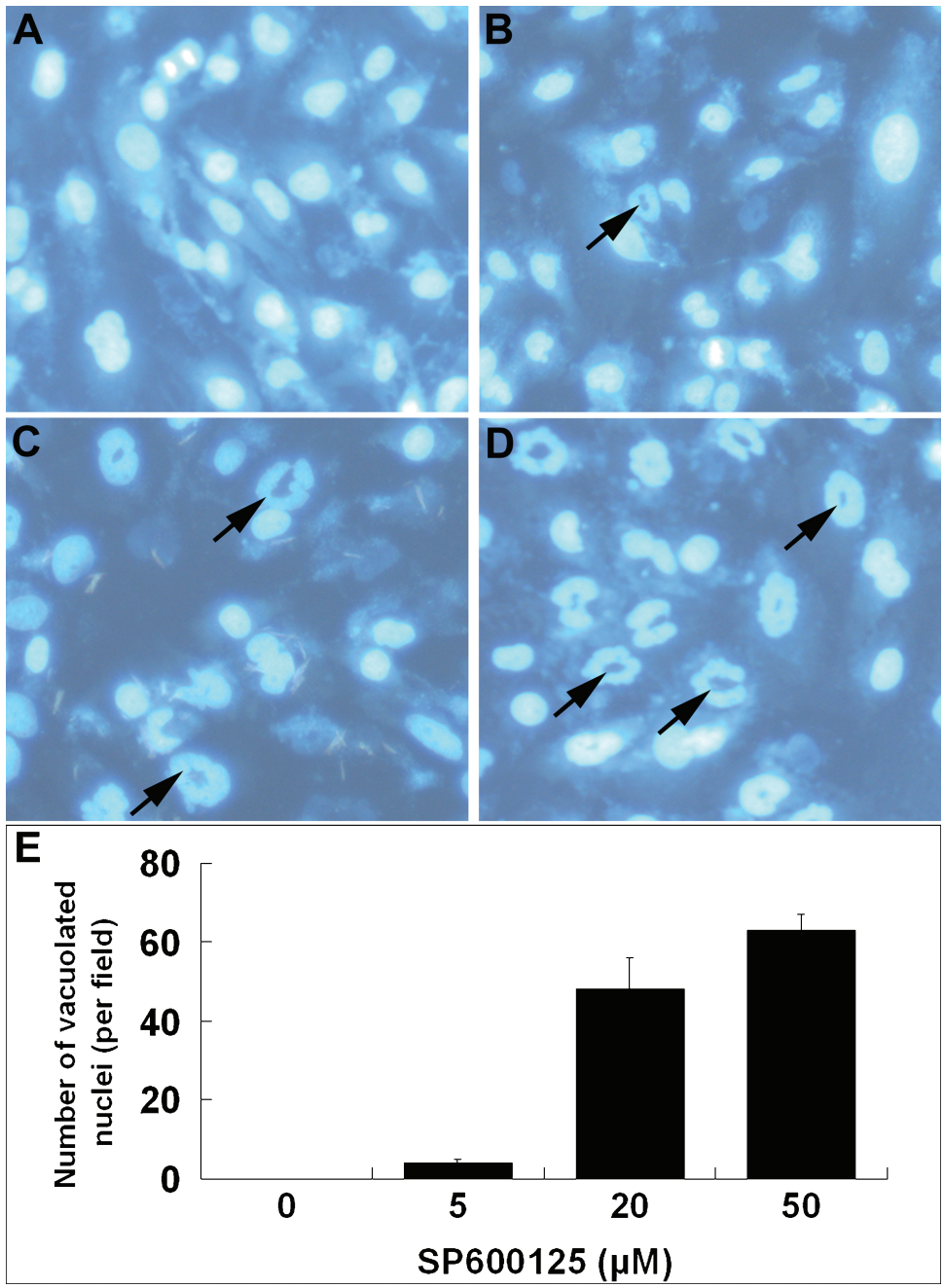

Fig. 4: SP600125 caused unique nuclear vacuolization in U251 cells. (A) DMSO treated cells were used as vehicle control and exhibited no nuclear vacuolization (200×). (B–D) Cells treated with 5, 20 or 50 μM SP600125 for 48 h, the vacuolated nuclei were pointed by black arrows (200×). (E) 10 random visual fields (200×) were selected in each sample and the average of vacuolated nuclei number in these fields was calculated. The number of cells with vacuolated nucleus increased in a concentration-dependent manner from 0 per field (vehicle control) to 4 ± 1 per field (5 μM) to 48 ± 8 per field (20 μM) and to 63 ± 4 per field (50 μM)

and phosphorylated-JNK (Thr183/Tyr185) were purchased from Cell Signaling Technology (Beverly, MA, USA).

4.6. Hoechst 33342 nuclear staining

U251 cells were determined by staining with Hoechst 33342 (Sigma–Aldrich, St. Louis, MO, USA), a DNA-specific fluorescent dye. Morphological changes in the nuclear chromatin of cells were monitored with a fluorescent microscope (Nikon Eclipse 800, Nikon Instrument, Inc., USA) equipped with a UV filter after staining the cells with Hoechst 33342 dye. Briefly, untreated and treated cells (1×106 cell/ml) were collected, washed with PBS, incubated with 10 μg/ml Hoechst 33342 for 15 min at 37 °C, mounted on glass slides and then observed under a microscope.

To count the number of vacuolated nuclei, 10 random visual fields (200 ×) were selected from each sample and the average of vacuolated nuclei number in these fields was calculated.

4.7. Statistical analysis

All data were analyzed by Student-Newman-Keul's test, and expressed as mean ± standard deviation (SD). Results at $P < 0.05$ were considered significant.

References

Bennett BL, Sasaki DT, Murray BW, O'Leary EC, Sakata ST, Xu W, Leisten JC, Motiwala A, Pierce S, Satoh Y, Bhagwat SS, Manning AM, Anderson DW (2001) SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase. Proc Natl Acad Sci USA 98: 13681–13686.

Bogoyevitch MA, Arthur PG (2008) Inhibitors of c-Jun N-terminal kinases: JuNK no more? Biochim Biophys Acta 1784: 76–93.

Bogoyevitch MA, Ngoei KR, Zhao TT, Yeap YY, Ng DC (2010) c-Jun N-terminal kinase (JNK) signaling: recent advances and challenges. Biochim Biophys Acta 1804: 463–475.

Duan W, Wong WS (2006) Targeting mitogen-activated protein kinases for asthma. Curr Drug Targets 7: 691–698.

Hamza MS, Reyes RA, Izumiya Y, Wisdom R, Kung HJ, Luciw PA (2004) ORF36 protein kinase of Kaposi's sarcoma herpesvirus activates the c-Jun N-terminal kinase signaling pathway. J Biol Chem 279: 38325–38330.

Jeong YM, Oh MH, Kim SY, Li H, Yun HY, Baek KJ, Kwon NS, Kim WY, Kim DS (2010) Indole-3-acetic acid/horseradish peroxidase induces apoptosis in TCCSUP human urinary bladder carcinoma cells. Pharmazie 65: 122–126.

Yi L, Ji XX, Lin M, Tan H, Tang Y, Wen L, Ma YH, Su Q (2010) Diallyl disulfide induces apoptosis in human leukemia HL-60 cells through activation of JNK mediated by reactive oxygen. Pharmazie 65: 693–698.

Yoon IS, Chung JH, Hahm SH, Park MJ, Lee YR, Ko SI, et al. (2011) Ribosomal protein S3 is phosphorylated by Cdk1/cdc2 during G2/M phase. BMB Rep 44: 529–534.