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Caspase-8 and p38MAPK in DATS-induced apoptosis of human CNE2 cells

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Abstract

Nasopharyngeal carcinoma is a common malignancy in Southern China of uncertain etiologic origin. Diallyl trisulfide (DATS), one of the major components of garlic (*Allium sativum*), is highly bactericidal and fungicidal. In this study, we investigated the function of p38 mitogen-activated protein kinase (MAPK) and caspase-8 in DATS-induced apoptosis of human CNE2 cells using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], flow cytometry assay, and Western blotting. After CNE2 cells were treated with DATS (50, 100, or 150 µM) for 24 h, cell viability rates were 75.9, 63.4 and 39.6%, and apoptosis rates were 24.5, 36.9, and 62.4%, respectively. The data showed that DATS induced CNE2 cell death in a dose-dependent manner. After human CNE2 cells were treated with 100 µM DATS and inhibitors (10 µM SB203580 and Z-LETD-FMK for p38MAPK and caspase-8, respectively), changes in cell viability and apoptosis and in p38MAPK and caspase-8 activity were detected. Cell viability rates were 66.5 and 68.1% and decreased 9.9 and 11.5% compared with inhibitor treatment alone. Apoptosis rates were 31.53 and 29.98% and increased 9.1 and 10% compared with inhibitor treatment alone. The results indicated that DATS activates p38MAPK and caspase-8, but both inhibitors have an effect on P38MAPK and caspase-8 activity. In conclusion, our data indicate that p38MAPK and caspase-8 are involved in the process of DATS-induced apoptosis in human CNE2 cells and interact with each other.

Key words: DATS; p38 MAPK; Caspase-8; Inhibitor; Apoptosis

Introduction

Mitogen-activated protein kinase (MAPK), an important intracellular signal transduction system, has a marked effect on the regulation of gene expression and cytoplasmic functional activities (1-3). The p38 signaling pathway is an important branch of the MAPK pathway, playing a significant role in a variety of physiological and pathological processes, such as inflammation, cell stress, apoptosis, cell cycle and growth, and so on (4,5). Caspase is an inactive enzyme zymogen under normal circumstances, but once activated it will trigger the caspase cascade, eventually leading to apoptosis. In the central control and effective stage of apoptosis, activated caspase-8 can lead directly to the appearance of apoptotic structural characteristics in cells, and play a key role in the process of apoptosis (6,7).

Nasopharyngeal carcinoma (NPC) is a malignant tumor of high incidence in the Southeastern region of Asia. In clinical practice, NPC is treated by radiation, but the therapeutic effect is not satisfactory (8-10). In recent years, many investigations have focused on extracting active ingredients from natural plants to prevent and treat cancer and to investigate their anti-cancer mechanism (11,12). Garlic (*Allium sativum*) is a common plant used mainly as a food and is considered to have medicinal properties in many cultures (13). Allicin is a general name for the main bioactive component of garlic, which is a compound of a variety of allyl sulfides (14-17). Diallyl trisulfide (DATS), the major component of allicin, has anti-inflammatory and antibacterial functions. However, DATS is an unstable compound that can be easily converted to diallyl disulfide during the extraction process due to external factors. Studies have shown that DATS can inhibit the growth of human tumor cells such as prostate cancer cells and gastric cancer cells (18-22). In the present study, we used SB203580, a p38MAPK inhibitor, and Z-LETD-FMK, a caspase-8 inhibitor, to determine the relation of p38MAPK and caspase-8 in the apoptosis process induced by DATS. We observed

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that p38MAPK and caspase-8 are involved in the process of DATS-induced apoptosis in human CNE2 cells and interact with each other.

Material and Methods

Material

DATS (99% purity) was purchased from Chia-tai Tianqing Pharmaceutical Co., Ltd. (China). RPMI1640, BSA and SB203580 were purchased from Sigma (USA). Z-LETD-FMK was purchased from Biovision (USA), goat horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody was purchased from Santa Cruz Biotechnology (USA). Antibodies to p38, phospho-p38 (p-p38), and caspase-8 were purchased from Cell Signaling (USA).

Cell culture

CNE2, a human NPC cell line, was provided by the Xiangya School of Medicine and cultured in RPMI1640 containing 10% heat-inactivated fetal bovine serum (FBS), benzylpenicillin (100 kU/L) and streptomycin (100 mg/L) at 37°C in an incubator containing humidified air with 5% CO_2.

Cell viability assay

Cells were seeded onto 96-well plates at 1 x 10^4 cells per well 24 h before treatment. The cultures were then rinsed in phenol-free RPMI1640 medium and incubated with the respective test substances in phenol-free and serum-free RPMI1640 for 24 h. In the inhibition test, cells were treated with DATS after being treated with inhibitors for 30 min. At the end of this time, 20 µL (5 mg/mL) MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was added to each well, and after incubation at 37°C for 4 h the MTT solution was removed and 200 µL dimethylsulfoxide (DMSO) was added to dissolve the crystals. The absorbance of each well at 570 nm was measured.

Flow cytometry analysis

Cells (12 x 10^6) were seeded into 100-mL cell culture bottles 24 h before treatment and then treated as described above and incubated for 24 h. Cells were then collected and single cell suspensions were prepared and centrifuged at 800 *g* for 5 min. The supernatant was discarded and cells were washed three times with cool PBS and fixed for 24 h with cool alcohol at 4°C. One-milliliter cell suspension (10^6/mL) was washed three times with cool PBS, treated with RNase for 30 min at 37°C, stained with propidium iodide (PI) for 30 min at 37°C in the dark, and used for flow cytometry analysis.

Western blotting

Cells in the logarithmic growth phase were treated as described above and incubated for 24 h. After fragmentation on ice for 20 min, the lysates were centrifuged at 15,000 *g* for 10 min at 4°C, the protein was collected, quantitated by the bicinchoninic acid (BCA) method, electrophoresed and isolated by 10% SDS-PAGE using the electrotransfer method, blocked, and hybridized on cellulose nitrate film. The protein expression of the cells was detected using the ECL Western blotting method. The densities of protein bands were calculated using the Quantyone software.

Statistical analysis

Data are reported as means ± SD of three independent experiments and were evaluated by one-way analysis of variance (ANOVA). Differences were considered to be significant at $P < 0.05$.

Results

Changes in cell activity

We tested the effect of DATS on human CNE2 cells. The cells were treated with various concentrations of DATS for 24 h and cell viability was determined by the MTT assay. As shown in Figure 1, 50 µM DATS induced a 75.9% decrease in cell viability. When the cells were incubated with 100 and 150 µM DATS, cell viability decreased by about 63.4 and 39.6%, respectively, at 24 h. DATS had a dose-dependent effect.

The MTT conversion assay was used to test the effects of inhibitors on the viability of CNE2 cells. After treatment with 10 µM SB203580 or Z-LETD-FMK for 30 min, 100 µM DATS induced a 66.5 or 68.1% decrease in cell viability at 24 h. When the cells were incubated with 10 µM SB203580

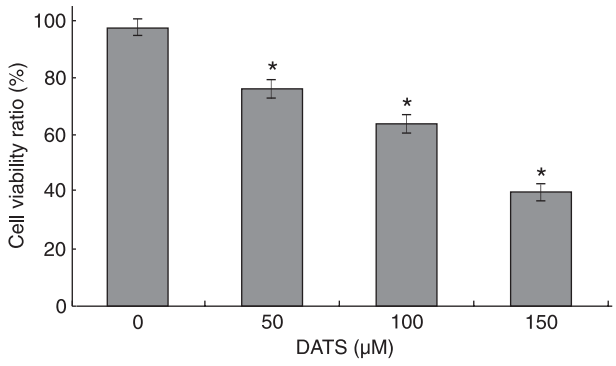

Figure 1. Effect of diallyl trisulfide (DATS) on cell viability. CNE2 cells were treated with 0, 50, 100, 150 µM DATS for 24 h. Cell viability was determined by MTT assay. Data are reported as means ± SD of three independent experiments. *P < 0.05 compared to control (one-way ANOVA).

or Z-LETD-FMK, cell viability decreased by about 76.4 or 79.6% at 24 h (Figure 2).

Flow-cytometry analysis of apoptosis

To further examine the effects of DATS on apoptosis, flow cytometry was used to quantify the apoptotic state. We found that 24.5, 36.9, and 62.4% of CNE2 cells became apoptotic when they were exposed to 50, 100, 150 μM DATS for 24 h (Figures 3 and 4). These results supported the view that DATS induces apoptosis of CNE2 cells in a concentration-

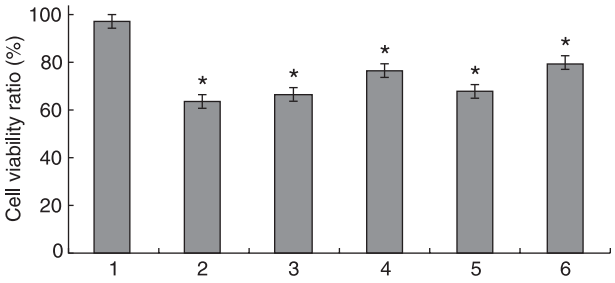

Figure 2. Effect of inhibitors on cell viability. *Lane 1*, Control; *lane 2*, cells treated with diallyl trisulfide (DATS; 100 μM); *lane 3*, cells treated with DATS (100 μM) after being treated with SB203580 (10 μM) for 30 min; *lane 4*, cells treated with SB203580 (10 μM); *lane 5*, cells treated with DATS (100 μM) after being treated with Z-LETD-FMK (10 μM) for 30 min; *lane 6*, cells treated with Z-LETD-FMK (10 μM). Data are reported as means ± SD of three independent experiments. *P < 0.05 compared to control (one-way ANOVA).

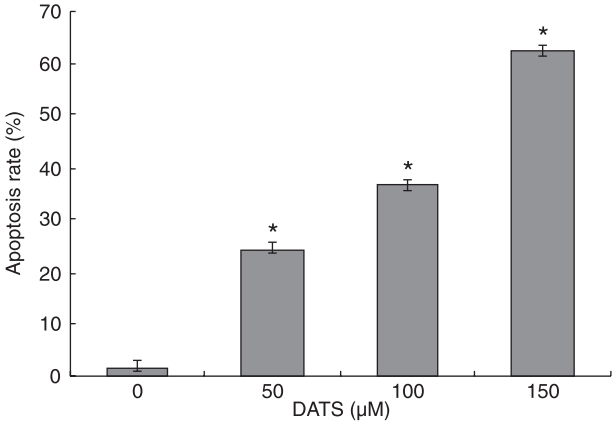

Figure 3. Apoptosis of CNE2 cells treated with diallyl trisulfide (DATS). CNE2 cells were treated with 0, 50, 100, 150 μM DATS for 24 h. Apoptosis was determined by flow cytometry. Data are reported as means ± SD of three independent experiments. *P < 0.05 compared to control (one-way ANOVA).

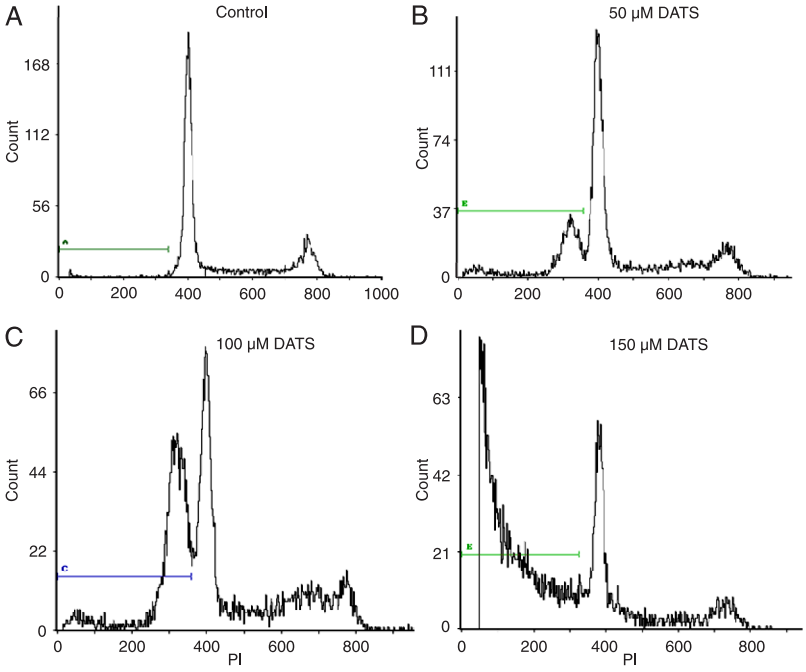

Figure 4. Effect of diallyl trisulfide (DATS) on apoptosis of CNE2 cells. *A*, Control; *B*, *C*, and *D*, CNE2 cells were treated with 50, 100, and 150 μM DATS, respectively. The results are representative of three independent experiments. PI = propidium iodide.

dependent manner.

We also determined the effects of both inhibitors on apoptosis by flow cytometry. Combined treatment with SB203580 plus DATS (100 µM) increased the proportion of apoptotic CNE2 cells by an absolute value of 9.1% when compared with inhibitor treatment alone. CNE2 cell apoptosis was increased by 10% with Z-LETD-FMK plus DATS (100 µM) compared with inhibitor treatment alone (Figures 5 and 6).

Protein expression

We tested the effects of DATS on p-p38 and caspase-8 activity as a function of dose by Western blotting. As shown in Figure 7, p-p38 and caspase-8 were markedly activated in a dose-dependent manner, with an accumulation of both after 24 h of DATS treatment. These data show that p-p38 and caspase-8 may play important roles in DATS-induced CNE2 cell apoptosis.

To test whether inhibitors block DATS-induced activation of p38 and caspase-8 in CNE2 cells, we also examined protein expression by Western blot analysis. When SB203580 or Z-LETD-FMK was added to CNE2 cells 30 min prior to DATS treatment, the activation of p-p38 and caspase-8 was markedly decreased. Similar results were observed with inhibitor treatment alone (Figure 8).

Discussion

Apoptosis is a type of physiological cell death that maintains body stability. The apoptosis process is strictly controlled by multiple genes, and malignant cell transformation is due, to a large extent, to the dysfunction of cell proliferation and death (23). The MAPK signal transduction pathway is an important signal transduction system for mediating extracellular stimulation of an intracellular response. A variety of extracellular stimuli can cause the phosphorylation chain reaction of the MAPK system, and this reaction can regulate cell proliferation, differentiation, apoptosis, and interactions (24,25). In recent years, stud-

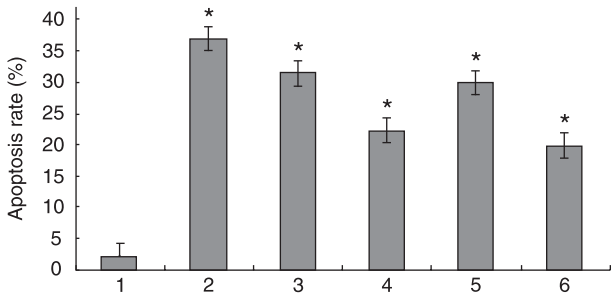

Figure 5. Apoptosis of CNE2 cells according to each treatment. *Lane 1*, Control; *lane 2*, cells treated with diallyl trisulfide (DATS) (100 µM); *lane 3*, cells treated with DATS (100 µM) after being treated with SB203580 (10 µM) for 30 min; *lane 4*, cells treated with SB203580 (10 µM); *lane 5*, cells treated with DATS (100 µM) after being treated with Z-LETD-FMK (10 µM) for 30 min; *lane 6*, cells treated with Z-LETD-FMK (10 µM). Data are reported as means ± SD of three independent experiments. *P < 0.05 compared to control (one-way ANOVA).

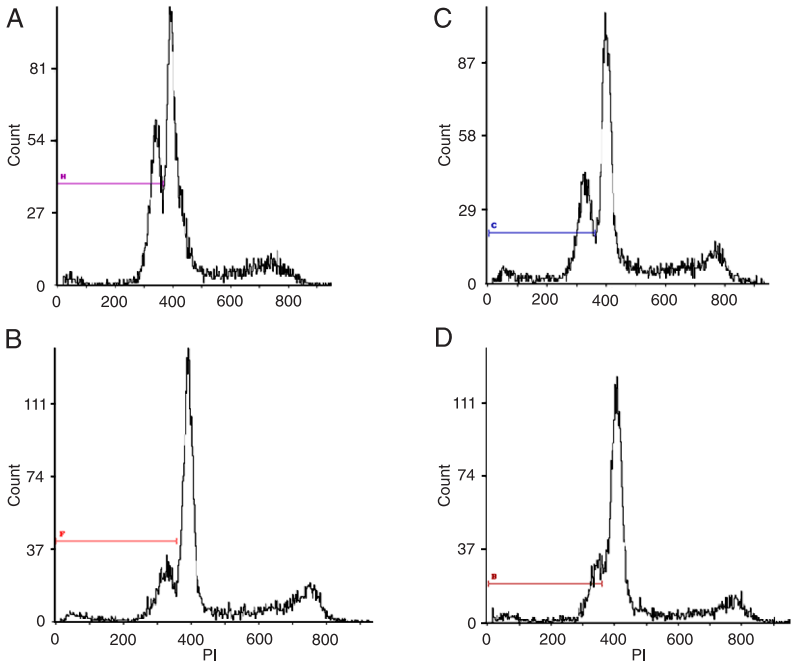

Figure 6. Effect of inhibitors on diallyl trisulfide (DATS)-induced apoptosis of CNE2 cells. *A*, Cells treated with DATS (100 µM) after being treated with SB203580 (10 µM) for 30 min; *B*, cells treated with SB203580 (10 µM); *C*, cells treated with DATS (100 µM) after being treated with Z-LETD-FMK (10 µM) for 30 min; *D*, cells treated with Z-LETD-FMK (10 µM). The results are representative of three independent experiments. PI = propidium iodide.

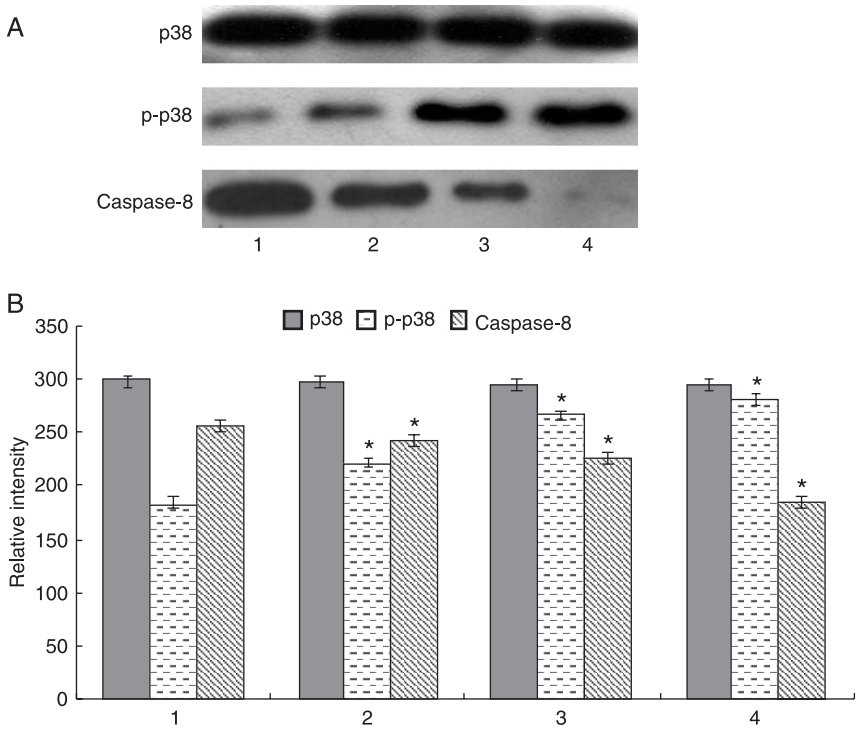

Figure 7. Effect of diallyl trisulfide (DATS) on protein expression determined by Western blotting. *A*, CNE2 cells were treated with 0 (lane 1), 50 (lane 2), 100 (lane 3), and 150 μM p-p38 DATS for 24 h. *B*, Representative graphs of Western blot densities. Data are reported as means ± SD of three independent experiments. *P < 0.05 compared to control (one-way ANOVA).

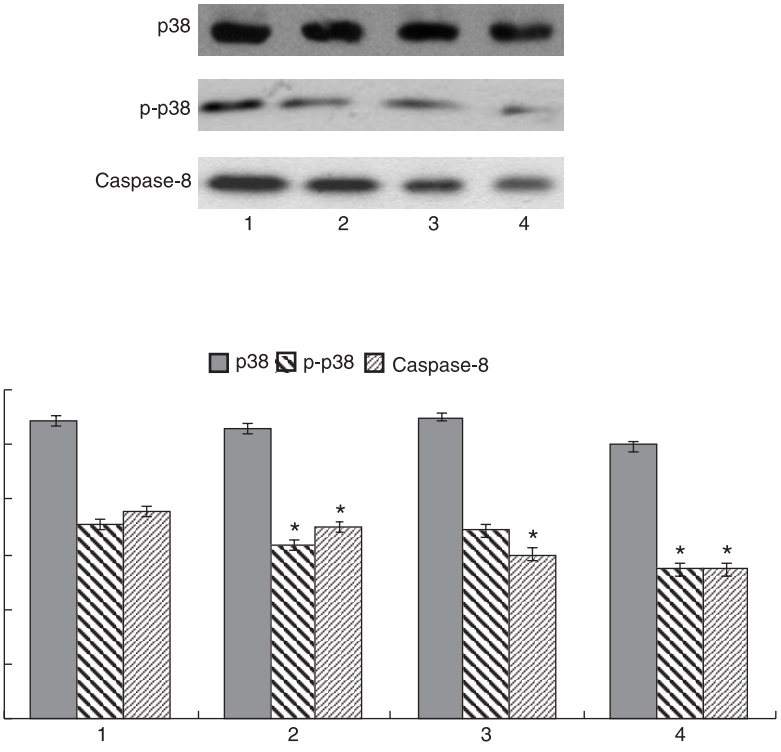

Figure 8. Effect of inhibition on protein expression determined by Western blotting. *A*, *Lane 1*, cells treated with diallyl trisulfide (DATS) (100 μM) after being treated with SB203580 (10 μM) for 30 min; *lane 2*, cells treated with SB203580 (10 μM); *lane 3*, cells treated with DATS (100 μM) after being treated with Z-LETD-FMK (10 μM) for 30 min; *lane 4*, cells treated with Z-LETD-FMK (10 μM). *B*, Representative graphs of Western blot densities. Data are reported as means ± SD of three independent experiments. *P < 0.05 compared to treatment with DATS + SB203580 (one-way ANOVA).

ies have shown that the apoptosis signal transduction is closely related to the activation of caspase, whose family exist in mammalian cells, with 16 members of the family having been identified thus far. The caspase enzyme family is a central effector of apoptosis. The undynamic caspase will trigger apoptosis when it is activated, and play a very important role as the central effector of apoptosis when the cells start the apoptotic process (26-30).

To probe the relationship between p38MAPK and caspase-8 in the apoptosis process of human CNE2 cells induced by DATS, we pretreated CNE2 cells with SB203580 and Z-LETD-FMK, phosphospecific inhibitors of p38 and caspase-8, and then added DATS. The results presented in the present study established a potential role for inhibitors of p38MAPK and caspase-8 in DATS-induced apoptosis. First, the inhibitors (SB203580 or Z-LETD-FMK) showed inhibitory activity on p38MAPK and caspase-8. Second, a combined treatment with DATS and inhibitors (SB203580 or Z-LETD-FMK) reduced the inhibition and the apoptotic activity of CNE2 cells increased compared to cells treated with DATS alone (Figures 1, 2, 3, 5, 7, and 8). The combined effect suggests a co-chemocytotoxic activity against human NPC. In conclusion, our results show that p38MAPK and caspase-8 are involved in the process of DATS-induced apoptosis in human CNE2 cells and interact with each other.

At present, some progress has been made regarding the effect of the MAPK signaling pathway on cellular apoptosis, but in-depth study is still needed to fully reveal its mechanisms of action (31,32). Our results show that the apoptosis process is strictly controlled by multiple genes and that p38MAPK and caspase-8 are involved in the process of DATS-induced apoptosis in human CNE2 cells, enhance DATS-induced apoptosis and interact with each other, but the mechanism involved needs further investigation. Further studies on the relationship between the MAPK signal transduction pathway and caspase in the cellular apoptosis process will lead to better clarification of the mechanism of apoptosis and will have important significance for the investigation of the anti-tumor mechanisms of DATS and for the design of new drugs.

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