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Effect of *Allium sativum* (garlic) diallyl disulfide (DADS) on human non-small cell lung carcinoma H1299 cells

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Abstract. This study was undertaken to elucidate the effect of diallyl disulfide from *Allium sativum*, an oil-soluble organosulfur compound found in garlic, in suppressing human non-small cell lung carcinoma H1299 cells. A potent increase in apoptotic cells has accompanied 1) a decrease in cell viability, 2) an increase of the fraction of G2/M-phase cells by up to 48.80 %, and 3) a transient increase of the phospho-p42/44 (phosphorylated p42/44 MAPK) in a time- and concentration-dependent manner. These results indicated that diallyl disulfide could induce apoptosis in human non-small cell lung carcinoma H1299 cells via, at least partly, G2/M-phase block of the cell cycle, related to a rise in MAPK phosphorylation.

INTRODUCTION

Diallyl disulfide (DADS) from garlic (*Allium sativum*) has been shown to have an antiproliferative effect on human tumor cells including those of colon, lung, skin, breast and liver origins (Sundaram & Milner, 1996; Hong *et al.*, 2000; Nakagawa *et al.*, 2001; Kwon *et al.*, 2002; Wen *et al.*, 2004; Druesne-Pecollo *et al.*, 2006; Tsai *et al.*,2007). Although the role and mechanism of DADS as an anti-cancer agent have not been completely elucidated, DADS has been demonstrated to induce cell-cycle arrest (G2/M block) and apoptosis in some cultured cell lines, such as leukemia HL-60 (Kwon *et al.*, 2002), neuroblastoma SH-SY (Filomeni *et al.*, 2003), and human nasopharyngeal carcinoma CNE2 cells (Zhang *et al.*, 2006).

A number of reports have described activation of the components of the MAPK pathway during apoptosis induced by Taxol, inducible nitric oxide synthase (iNOS) or DADS (Iwai-Kanai *et al.*, 2002; Wen *et al.*, 2004; Zhang *et al.*, 2006). The MAPK pathway is central to the control of growth, differentiation, and survival (Pouyssegur & Lenormand, 2003). It is activated in response to a diverse range of stimuli including growth factors, hormones, neurotransmitters, and cell stressors. So far, c-Jun NH2-terminal kinases (JNKs), p38-MAPK, AKT, and p42/44-MAPK have all been identified as MAPK subfamilies. p38-MAPK is more commonly activated in response to cytokines, stress, and cellular damage while p42/44-MAPK is activated by growth stimuli. Both are generally considered to be pro-survival mediators (Flamigni *et al.*, 2001). Our previous reports showed that both p38-MAPK and p42/44-MAPK produced cytoprotective effects in human hepatoma cells (Wen *et al.*, 2004) and DADS could induce apoptosis in human nasopharyngeal carcinoma CNE2 cells via, at least partly, S-phase block of the cell cycle, related to a rise in MAPK phosphorylation (Zhang *et al.*, 2006). However, the role of MAPKs in inducing tumor apoptosis on human non-small cell lung carcinoma H1299 cells is not

entirely clear (Amato *et al.*, 1998; Lee et al., 1998). In this report, we find that DADS induces cell cycle arrest and transient phosphorylation of p42/p44–MAPK, while inducing apoptosis in human non-small cell lung carcinoma H1299 cells.

MATERIALS AND METHODS

Materials

Diallyl disulfide from *A. sativum* (80% purity) was purchased from Fluka Co. BSA and Hoechst 33258 were purchased from Sigma Chemical Co. DMEM medium was purchased from GIBCO. Specific anti-phospho- and the cocktail of anti-total-p42/44 antibodies, and goat horseradish peroxidase (HRP)-conjugated secondary antibody were purchased from Cell Signaling. The Phototope-HRP Western Detection Kit was purchased from New England Biolabs.

Cell culture

Human non-small cell lung carcinoma H1299 cell line, was provided by Memorial Sloan-Kettering Cancer Center (New York, USA) and was cultured in DMEM with 10% heat-inactivated fetal bovine serum (FBS), benzylpenicillin (100 kU/l), and streptomycin (100 mg/l) at 37 ºC in humidified air with 5% CO_2. Chang liver cells were supplied by Xiangya Hospital, Central South University (Changsha, China).

Cell viability assay

To assess the cytotoxic effects of DADS in H1299 cells, we used a tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay (Xiao *et al.*, 2006). In this assay, MTT is used as a colorimetric substrate for measuring cell viability. When cells are injured there is an alteration in cellular redox activity such that cells are unable to reduce the dye. Cells were plated in 24-well plates and grown to 80% confluence. The cultures were then rinsed in phenol-free DMEM media and incubated with specific test substances in phenol- and serum-free DMEM for 24 h. At the end of this time, MTT was added to a final concentration of 0.5 g/l. After 1 h incubation, cultures were removed from the incubator and the formazan crystals were solubilized by adding 10% (v/v) Triton X-100 and HCl 0.1 mol/l in isopropanol equal to the volume of original culture media. Colorimetric determination of the reduced MTT was measured at 570 nm. Chang liver cells were used as control.

Cell cycle analysis

H1299 cells were grown to 60% confluence and the culture media was changed to a complete medium with final concentrations of 0, 20, 50, and 100 µmol/l DADS, and then continued for 24 hr. Afterwards, cells were harvested and fixed in 70% ethanol at 4 ºC for 24 hr. Immediately before analysis the fixed cells were treated with RNase (100 mg/l) at 37º for 30 min, and then propidium iodide (10 mg/l) added. After incubating at 4 ºC for 30 min in darkness, samples (3×10^4 cells for each assay) were analyzed for DNA content using an Epics ALTRA (Beckman-Coulter) flow cytometer and proprietary software.

Apoptosis analysis

The Hoechst 33258 staining method was used to detect apoptosis induced by DADS. After treatment with or without DADS in phenol- and serum-free DEME for 24 hr, morphological changes of H1299 cells were observed by fluorescence microscopy after Hoechst 33258 staining. Apoptosis was routinely determined by counting the numbers of cells with condensed or fragmented chromatin as described previously (Bates *et al.*, 1994). Six randomly chosen fields of view were examined with a minimum number of 500 cells scored for each condition.

Analysis of cell cycle and quantification of apoptosis

Flowcytometric analysis of H1299 cells was performed using a FAScan for 24 h of culture. Thereafter, cells were washed twice with PBS solution. Then, the cell suspension was centrifuged at 700 rpm, for 5 min at room temperature. Decanting of all the supernatant were followed by adding 1mL of 70%

methanol to the pellet. After incubation at –20 °C for at least 24 h, prior to the samples being analyzed by the flow cytometry (Beckman-Coulter), 1mL of cold propidium iodide (PI) stain solution (50 µg/ml PI, and 20 µg/ml RNase A) was added to the mixture and it was incubated for 15 min in darkness at room temperature. Propidium iodide was excited at 488 nm and fluorescence signal was subjected to logarithmic amplification with PI fluorescence (red) being detected above 600 nm. Cell cycle distribution is presented as the number of cells *versus* the amount of DNA as indicated by the intensity of fluorescence, and the extent of apoptosis was determined by counting cells of DNA content below Sub G1, G0/G1, S and G2/M phases with CELL Quest Version 3.3 software. The percentage of hypodiploid cells (SubG1 phase) over total cells was calculated and expressed as percent of apoptosis.

Preparation of lysates

For MAPK detection, H1299 cells cultured in 6-well culture plates were grown to 80-90% confluence, then nutrient-starved for 24 hr in serum-free DMEM. Various concentrations of DADS were added for the indicated times. After three washes with ice-cold PBS, cells were lysed with 60 µl of ice-cold lysis buffer containing NaCl (50 mmol/l), Na_3VO_4 (2 mmol/l), $Na_4P_2O_7$ (10 mmol/l), NaF (100 mmol/l), phenylmethyl-sulfonyl fluoride (1 mmol/l), DTT (2 mmol/l), and HEPES (50 mmol/l) at pH 7.5, along with Triton X–100 (0.01%), leupeptin 20 (50 µmol/l), and aprotinin 1×10^5 U/L. The lysates were obtained by centrifugation at 13,000 × g at 4 °C for 10 min and the concentration of total cell protein was determined by spectrophotometer (Bradford, 1976).

Western blot analyses

SDS sample buffer containing Tris–HCl 0.33 mol/l, SDS 8% (w/v), glycerol 40% (v/v), and bromophenol blue 0.4% was added to cell lysates. After boiling the extracted protein for 5 min, 20 µg was resolved by SDS–PAGE. The protein was transferred to a nitro-cellulose membrane which was then blocked at 25 °C for 1 hr with 5% BSA in TBST (Tris–HCl 20 mmol/l, pH 7.5, NaCl 137 mmol/l containing 0.1% Tween-20). The blots were incubated with the primary antibodies against phospho-p42/p44, or total-p42/p44 at 1:1000 dilutions at 4 °C overnight, followed by incubation at room temperature for 1 hr with secondary antibody (horseradish peroxidase conjugated) at 1:2000 dilution. Immunoreactive signals were visualized by the Phototope Western Detection System (New England Biolabs). As a protein loading control, two gels for each group were loaded in parallel with the same protein samples and blotted for activated/phosphorylated MAPKs or total MAPKs. Bands of MAPK were quantitatively determined by Quantity One version 4.5.2 (Biosoft).

Statistical analysis

Data are reported as means ± standard deviation of three independent experiments and evaluated by one-way analysis of variance (ANOVA). Significant differences were established at $p < 0.05$.

RESULTS

Cytotoxicity of DADS to H1299 cells

Cytotoxicity of DADS on human non-small cell lung carcinoma H1299 cells was measured by MTT assay. The cell in cultures was exposed to 0–100 µmol/l DADS for 24 h. DADS showed a dose-dependent (Fig. 1A) and time-dependent (Fig. 1B) inhibitory effect on the growth of H1299 cells in a 24-h exposure to DADS. The same treatment was performed on a normal cell line. However, the normal cells were less sensitive to cytotoxic effect of DADS than the H1299 cells (data not shown). Thereafter, we used the concentrations of 0, 20, 50, and 100 µmol/l in the following investigations.

Induction of apoptosis by DADS

To test whether DADS induced apoptosis in H1299 cells, DADS-treated H1299 cells were examined by fluorescence microscopy after staining with Hoechst 33258. After exposure to DADS (100 µmol/l) for 24 hr, H1299 cells showed typical morphologic changes of apoptosis: cell volume was reduced,

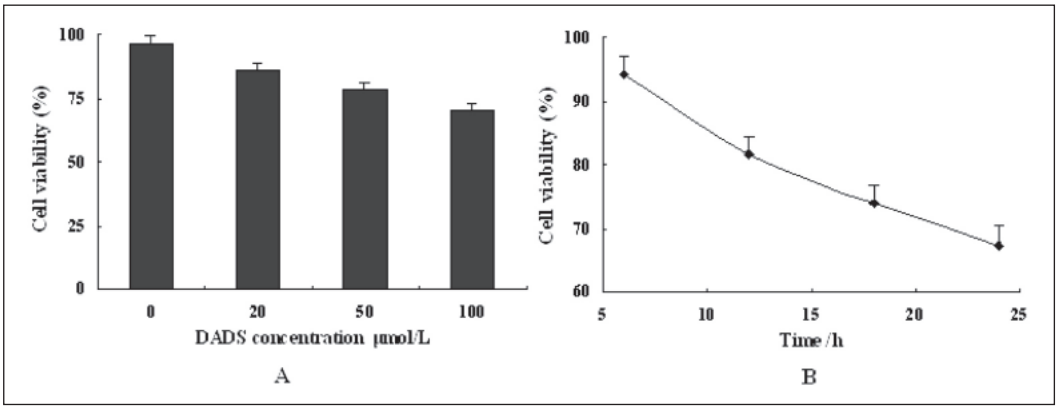

Figure 1. DADS showed a dose-dependent (A) and time-dependent (B) inhibitory effect on H1299 cells.

chromatin became condensed, and nuclei became fragmented (Fig. 2B), compared with untreated cells (Fig. 2A).

Similar to DADS-induced cytotoxicity, DADS also induces apoptosis of H1299 cells in a concentration-dependent manner. Treatment with 100 μmol/l DADS for 24 hr increased the number of apoptotic cells (measured by cell counting) to 19.28% compared with 1.79% in the untreated control, 4.92% when treated with 20 μmol/l, and 10.66% when treated with 50 μmol/l (Fig. 3).

Flowcytometric analysis of apoptosis and cell cycle distribution

To further examine the effect of DADS on apoptosis and cell cycle distribution, we used flow cytometry to quantify the apoptotic state (Figs. 4, and 5). We found that 10.7% and 18.5 % of H1299 cells became apoptotic (at the hypodiploid phase, also named subG1 phase) when they were exposed to 100 μmol/l DADS for 24 h (Fig. 4C and D), compared with 1.79% in the untreated control (Fig. 4A). The results indicated that DADS induced apoptosis in a concentration-dependent manner.

We detected cell cycle distribution with concentrations of DADS treatment of 0, 20, 50, and 100 μmol/l. As displayed in Fig. 5, the percentages of cells in G2/M-phase were 12.10%, 12.55%, 11.0%, and 48.80%, respectively. Percentages did not differ significantly during the G1-phase and S-phase. These results indicate that DADS

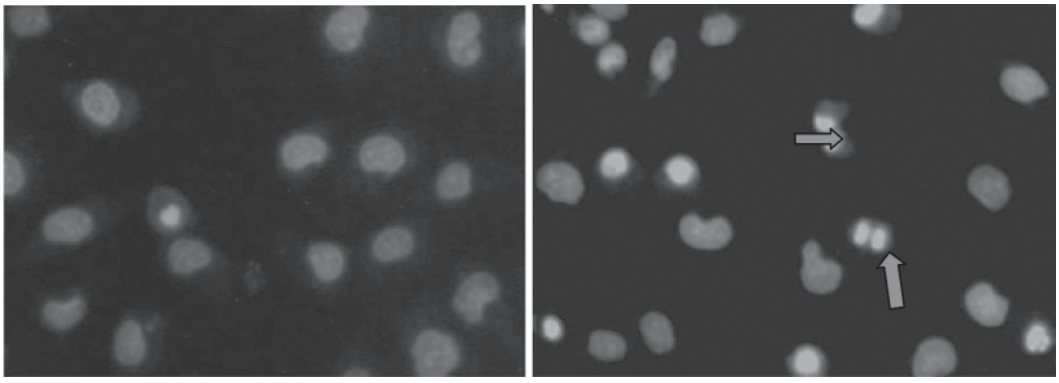

Figure 2. Apoptosis of H1299 cells were observed by Hoechst 33258 fluorescence staining.
A. Control group: normal nuclei; B. Treated with 100 μmol/l DADS for 24h: the nuclei of dead cells appear condensed. (Magnification × 600)

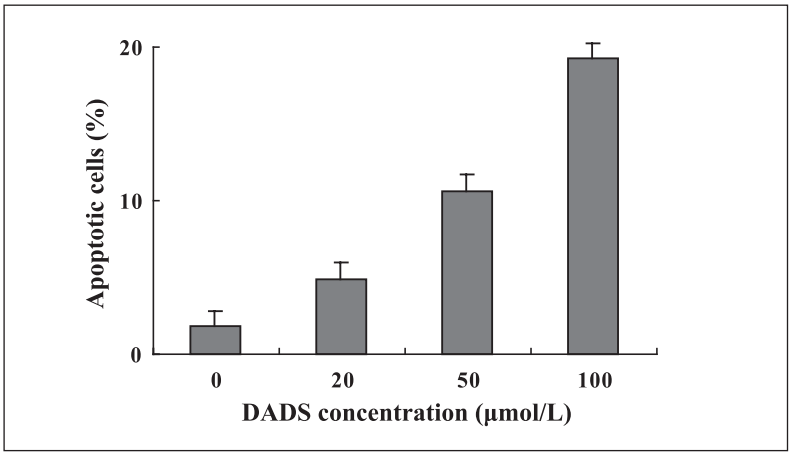

Figure 3. Cell apoptosis of H1299 cells treated with DADS.
H1299 cells were treated with 0, 20, 50, 100 μmol/l DADS for 24 h. After staining with Hoechst 33258, six randomly chosen fields of view were observed with a minimum number of 500 cells scored in each condition. Apoptotic cells (condensed with fragmented nuclei) were calculated as a ratio to the total number of cells counted. *P < 0.01 *vs* control (untreated).

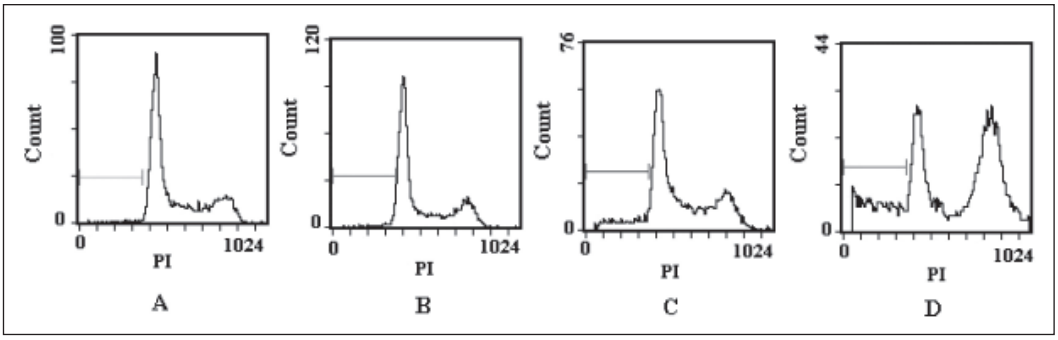

Figure 4. Effect of DADS induced apoptosis and cell cycle progress in H1299 cells.
Cultured cells were treated with or without DADS: (A) 0 μmol/l, (B) 20 μmol/l, (C) 50 μmol/l, and (D) 100 μmol/l. Twenty-four hours later, cell cycle was analyzed by flow cytometry. H1299 cells showed a sub-G1 peak, i.e., characteristic of apoptotic cells, which was more evident in 100 μmol/l DADS-treated group. PI is the relative fluorescence intensity.

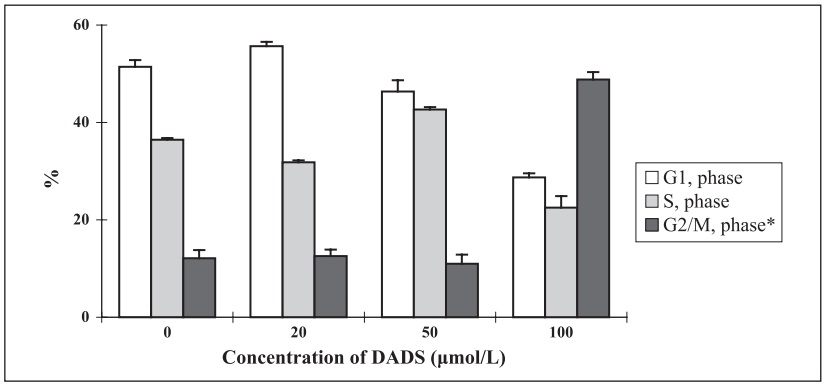

Figure 5. Effect of DADS on the cell-cycle distribution of H1299 cells by flow cytometry analysis. *P < 0.01 vs control.

induced cell cycle arrest in G2/M-phase in H1299 cells in a concentration-independent manner.

Transient increase in activation of endogenous MAPKs during DADS-induced H1299 cell apoptosis.

To directly address the involvement of MAPKs in DADS-induced apoptosis, phospho-p42/p44 proteins were assayed in DADS-treated H1299 cells using anti-phospho-p42/p44 antibody. As shown in Fig. 6, DADS caused a time-dependent activation of p42/p44-MAPK. DADS (100 µmol/l) stimulated the activation of p42/44-MAPK in a time-course with maximal induction at 60 to 120 min after stimulation. Treatment with DADS at concentrations of 0, 20, 50, 100 µmol/l for 10 min stimulated an increase in phospho-p42/44 in a concentration-dependent manner (Fig. 7). These results suggest that activation of phospho-p42/44 MAPKs plays a role in the mechanism of DADS-induced apoptosis.

DISCUSSION

Diallyl disulfide (DADS) is an oil-soluble sulfur compound from garlic that is produced as a result of decomposition of allicin. About 60% of garlic oil was reported to be DADS, indicating that it is the most prevalent oil soluble garlic constituent (Wu *et al.*, 2005). Apoptosis induction by DADS has been investigated in human colon, lung, prostate, leukemic, gastric, basal cell and mammary cancer cells *in vitro*. In the present study, we have found that DADS inhibits the growth of H1299 cells, as shown by decreases in cell viability. The growth inhibitory properties of DADS are likely to be attributable to its induction of apoptotic cell death as indicated in Figs. 1, 2, and 3.

Apoptosis induction by DADS involves different apoptotic genes and enzymes depending on the cell type. Generally, up-regulation of Bax, down regulation of Bcl-2 and Bcl-xL, cytochrome *c* release, activation of caspase-3 and caspase-9, increase of

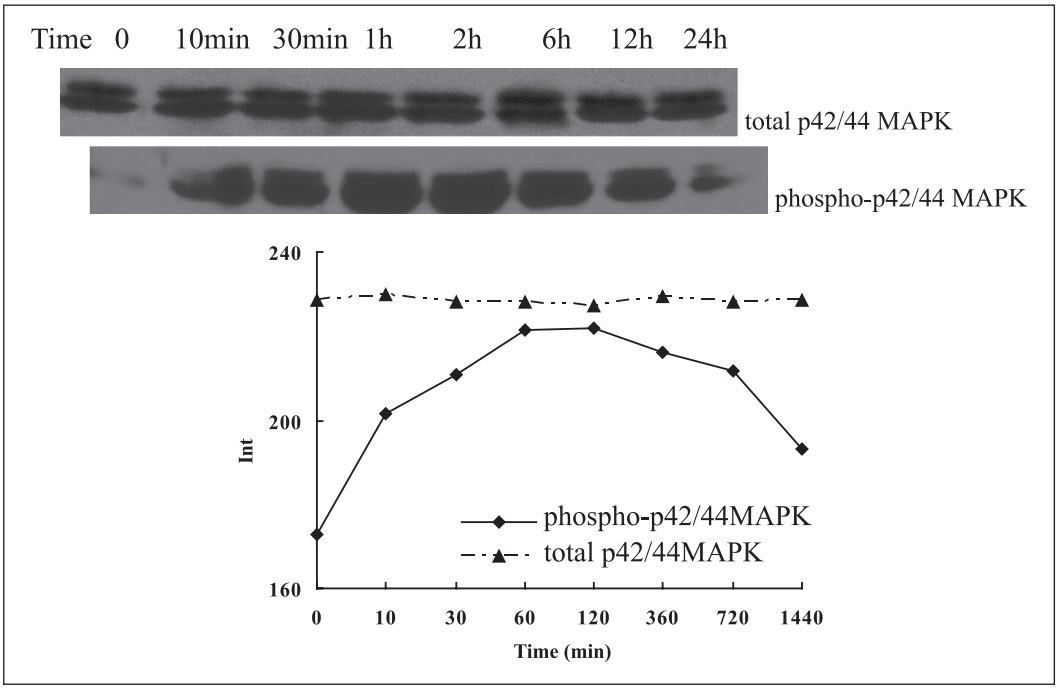

Figure 6. Time course of DADS-induced activation of p42/p44 MAPK in H1299 cells.
Cells were treated with 100 µmol/l DADS for 0-24 h, and Western blot analysis was performed. Extracts (60 µg of protein) from H1299 cells were resolved by SDS-PAGE and probed with anti-phospho-p42/44 antibody. These experiments were repeated three times with similar results, and typical data are presented. In parallel experiments, the amount of total p42/44 MAPK was determined in the same cell extracts with anti-total p42/44 MAPK antibody.

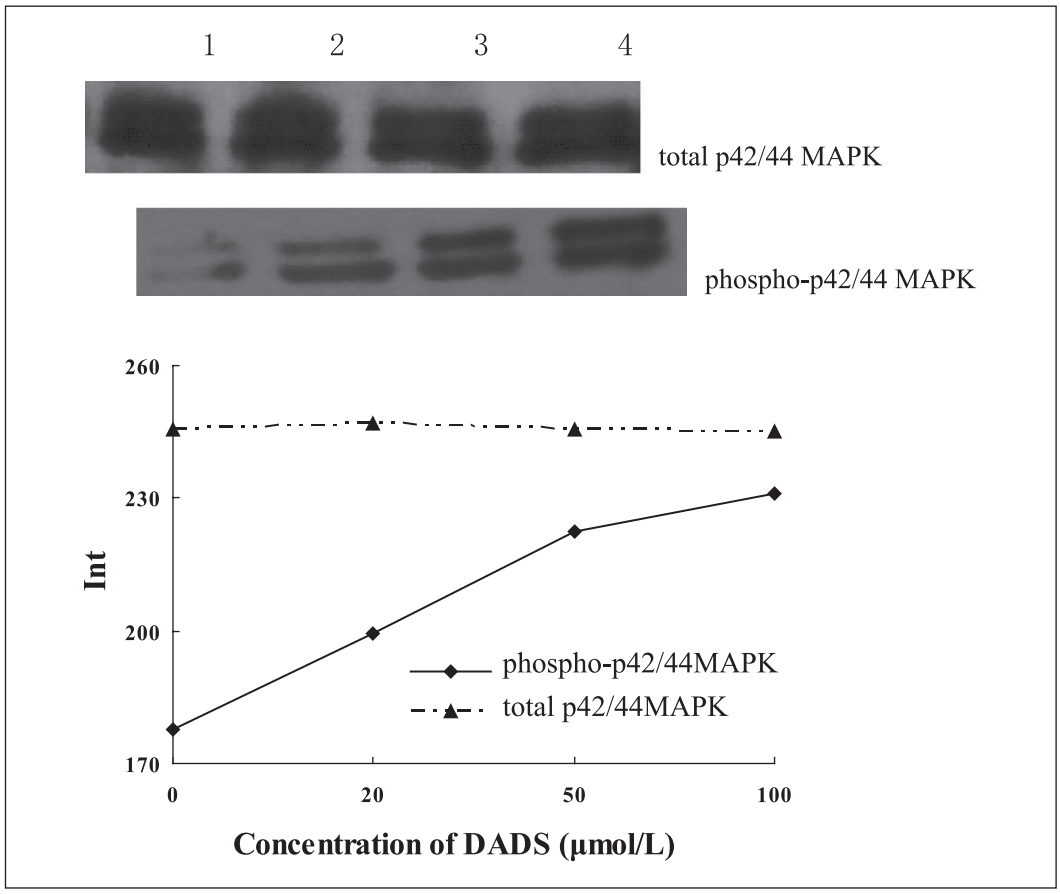

Figure 7. Concentration-dependent activation of p42/44 MAPK in H1299 cells.
H1299 cells were treated with increasing concentrations (0-100 μmol/l) of DADS for 1 h. Western blot assays were performed by applying 60 g of the extracts and probing with anti-phospho–p42/p44 or anti-total p42/p44 MAPK antibody after 10 min stimulation. Results were confirmed in triplicate. Lane 1: 0 μmol/l DADS, 2: 20 μmol/l DADS, 3: 50 μmol/l DADS, 4: 100 μmol/l DADS.

intracellular free calcium, cleavage of caspase-3 and PARP have been reported. These findings suggest that DADS-induced apoptosis is dependent on the classical mitochondria pathway.

A number of pathways are known to lead to apoptosis. Components of the apoptosis signaling cascade including caspases (Talanian *et al.*, 1997) along with several other triggers and regulators such as Fas ligand (FasL) (Sharma *et al.*, 2000) and Bcl-2 family members, are among the most promising targets for pharmacological modulation of cell death (Gross *et al.*, 1999).

The Bcl-2 family and caspase-3 are important regulators of apoptosis (Farrow *et al.*, 1996). The Bcl-2 proteins are associated with up-regulation of Bax and down-regulation of Bcl-xL. Other studies have suggested that induction of apoptosis might be through activation of the mitochondrial pathway involved in Bcl-2 down-regulated cytochrome release into the cytosol, and activation of caspase-9 and caspase-3 (Filomeni *et al.*, 2003). It has also been shown that DADS-induced apoptosis is mediated by activation of caspase-3 (Nakagawa *et al.*, 2001). In DADS-treated HL-60 cells, caspase-3 activation is evidenced by an increase in protease activity and proteolytic cleavage activity of the proenzyme

Excessive intracellular calcium is known to lead to apoptosis in several *in vitro* models (Hong *et al.*, 2000). In HCT–15 cells, DADS was found to cause a sustained and

dose-dependent increase in intracellular calcium. We have already described that DADS can activate the components of the MAPK pathway and phosphorylated MAPK inhibitors (SB203580 and U0126) cause apoptosis in human HepG2 hepatoma cells (Wen *et al*., 2004). Our present data demonstrate that DADS can transiently activate p42/p44-MAPK in a time- and concentration-dependent manner during DADS-induced apoptosis in H1299 cells (Figs 6 and 7). This suggests that DADS-induced phospho-p42/44 appears to play a role in maintaining the regulation of apoptosis in H1299 cells.

DADS has been demonstrated to induce cell cycle arrest in G2/M-phase in some cultured cell lines. The block at G2/M-phase might explain the increase in apoptosis through activation of the mitochondrial pathway (Filomeni *et al*., 2003). In our present study, the effects of DADS on the cell cycle shows that DADS also induces an increase in the number of H1299 cells in G2/M-phase reaching values of 48.80%. The percentage of the cell population showing apoptotic features was 19.28%, suggesting that the block in G2/M-phase finally results in triggering of the apoptotic program.

In summary, our results indicate that DADS can induce apoptosis in human non-small cell lung carcinoma H1299 cells via, at least partly, G2/M-phase block of cell cycle, related to a rise in MAPK phosphorylation. To this end, further studies on definitive mechanisms of the cancer chemotherapeutic activities of DADS in animal models, and even clinical trials are needed.

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