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• BRIEF REPORTS •

# Diallyl disulfide-induced G2/M arrest of human gastric cancer MGC803 cells involves activation of p38 MAP kinase pathways

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## Abstract

**AIM:** To determine the role of p38 MAP kinase signal transduction pathways in diallyl disulfide (DADS)-induced G2/M arrest in human gastric cancer MGC803 cells.

**METHODS:** MGC803 cell growth inhibition was measured by MTT assay. Phase distribution of cell cycle was analyzed by flow cytometry. Expression of Cdc25C, p38, phosphorylation of p38 (pp38) were determined by Western blotting.

**RESULTS:** MTT assay showed that SB203580, a specific p38 MAPK inhibitor blocked DADS-induced growth inhibition. Flow cytometry analysis revealed that treatment of MGC803 cells with 30 mg/L DADS increased the percentage of cells in the G2/M phase from 9.3% to 39.4% ($P<0.05$), whereas inhibition of p38 activity by SB203580 abolished induction of G2/M arrest by DADS. Western blotting showed that phosphorylation of p38 was increased 3.52-fold following treatment of MGC803 cells with 30 mg/L DADS for 20 min ($P<0.05$), whereas Cdc25C was decreased 68% following treatment of MGC803 cells with 30 mg/L DADS for 24 h ($P<0.05$). Decreased Cdc25C protein expression by DADS was attenuated by SB203580 ($P<0.05$).

**CONCLUSION:** DADS-induced G2/M arrest of MGC803 cells involves activation of p38 MAP kinase pathways. Decreased Cdc25C protein expression by p38 MAPK played a crucial role in G2/M arrest after treatment with DADS.

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## INTRODUCTION

Unlimited and uncontrolled cell proliferation is obviously characteristics of tumor cells[1,2]. Given that disruption of cell cycle plays a crucial role in cancer progression[3], its modulation by phytochemicals seems to be a logical approach in controlling carcinogenesis. Thus cell cycle regulation and its modulation by various natural (plant-derived) and synthetic agents are gaining widespread attention in recent years[4-18]. Most of the plant products with anticancer activity are strong antioxidants and some of them are effective modulators of protein kinase/phosphatases that are associated with cell cycle regulation. DADS is a major component of cooked garlic and oil-soluble organosulfur compound in processed garlic, which inhibits the proliferation of human breast, hepatoma, lung, bladder, colon cancer cells and human leukemia HL-60 cells[19-25]. Previous studies showed that the ability of DADS to suppress HCT-15, HT-29 cell proliferation was related to its propensity to induce a G2/M arrest[23,24]. However, the molecular mechanisms by which DADS exerts its effects on tumor cells leading to inhibition of cell growth and induction of G2/M arrest are largely unknown. p38 MAP kinase (p38) is a member of the mitogen-activated protein (MAP) kinase signaling cascade which has been shown to regulate a variety of cellular events such as cell proliferation, differentiation, and apoptosis[26-28], and may therefore be a potential target of DADS action. Gastric cancer is one of the most common malignant tumors in China[29-30]. Our previous studies showed that DADS could inhibit human gastric cancer MGC803 cell growth[31]. In this study, signaling pathways of p38 MAPK-induced G2/M arrest in DADS treated MGC803 cells were investigated for their involvement in the mechanisms of DADS-induced growth inhibition.

## MATERIALS AND METHODS

### *Materials*

Human gastric cancer cell line MGC803 was purchased from Cancer Research Institute of Hunan Medical University. DADS was purchased from Fluka Chemika (Ronkonkoma, NY). Monoclonal anti-p38, anti-pp38 antibodies and SB203580 were purchased from Cell Signaling (Beverly, MA). Polyclonal anti-Cdc25C antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

### *Methods*

**Cell culture and MTT assay** MGC803 cells were cultured in RPMI 1640 medium (Gibco) supplemented with 100 mL/L fetal bovine serum (Sijiqing Co.1 Hangzhou) with addition of 100 U/mL penicillin, 100 U /mL streptomycine. Cells in suspension (50 μL) were added to each well of a 96-well culture plate and incubated for 24 h at 37 °C in a humidified atmosphere of 50 mL/L $CO_2$ in air. The 96-well culture plate was divided into 4 sections, with one section being treated by culture media, the others were treated by one of the followings at 50 μL: culture media containing 2×reagents (10 μmol/L SB203580, 30 mg/L DADS, 30 mg/L DADS+10 μmol/L SB203580). In the latter group, SB203580 was added 1 h prior to DADS. The cultures were again incubated as above. After 72 h, 20 μL 5 g/L MTT solution was added to each well, and the cultures were further incubated in 100 μL DMSO solution. A microplate reader was used to measure absorbance at 570 nm for each well. Growth inhibition rate was calculated as follows: growth inhibition rate = (1-$A_{570/nm}$ of treated cells/ $A_{570/nm}$ of control cells)×100%.

**Cell cycle analysis** Cells were incubated in culture media alone or culture media containing reagents (10 μmol/L SB203580, 30 mg/L DADS, 30 mg/L DADS+10 μmol/L SB203580), at 37 °C for 24 h. In the latter group, SB203580 was added 1 h prior to DADS. Cells were harvested in cold PBS, fixed in 700 mL/L ethanol, and stored at 4 °C for subsequent cell cycle analysis. Fixed cells were washed with PBS once and suspended in 1 mL of PI staining reagents (20 mg/L ribonuclease and 50 mg/L propidium iodide). Samples were incubated in the dark for 30 min before cell cycle analysis. The distribution of cells in the cell cycle was measured by a flow cytometer.

**Western blotting** MGC803 cells treated with different reagents were harvested, rinsed twice with cold PBS, and incubated in the lysis buffer containing 50 mmol/L Tris (pH7.5), 150 mmol/L NaCl, 10 mL/L Np-40, 1 g/L SDS, 10 g/L sodium deoxycholate, 1 mmol/L DTT, 1 mmol/L PMSF, 2.5 mg/L leupeptin, 25 mg/L aprotinin on ice for 20 min. Following the centrifugation at 12 000 *g* for 30 min at 4 °C. The amount of protein in the supernatant was determined using BCA protein assay reagent. Equal amount of protein sample was completely vortexed with 2×SDS-gel buffer, and boiled for 5 min at 100 °C to dissolve the bound proteins. The samples were segregated on 100 g/L SDS-acrylamide gel, transferred onto a nitrocellulose membrane and blocked with 50 g/L defatted milk, then probed with different primary antibodies. Anti-mouse or anti-rabbit IgG conjugated peroxidase was as a secondary antibody. The filters were then incubated in SuperSignal ECL-HRP detection reagent for 1 min followed by exposure to X-ray film.

### *Statistical analysis*

Results were analyzed by SPSS10.0 statistical software. Data were expressed as mean±SD. Comparisons between different groups were made by one-way ANOVA (with LSD for post hoc analysis) or $\chi^2$ test. $P<0.05$ was taken as statistically significant.

## RESULTS

### *Effect of reagents on growth inhibition of MGC803 cells*

As shown in Table 1, 30 mg/L DADS suppressed MGC803 growth by 58.6% ($P<0.05$). SB203580 10 μmol/L alone slightly reduced cell growth, In contrast, SB203580 blocked DADS-induced growth inhibition ($P<0.05$). Thus, addition of SB203580 to the cells decreased the inhibitory ability of DADS to MGC803 cells growth.

**Table 1** $A_{570nm}$ of MGC803 cells exposed to reagents

| $n$ = 12 | Control | SB203580 (10 μmol/L) | DADS (30 mg/L) | DADS (30 mg/L) +SB203580 (10 μmol/L) |
|---|---|---|---|---|
| χ | 0.78 | 0.076 | 0.32 | 0.53 |
| *S* | 0.038 | 0.035 | 0.028 | 0.024 |
| IR (%) | | 3.4 | 58.6[a] | 31.7[a] |

[a]$P<0.05$ *vs* control; IR: inhibition rate.

**Table 2** Distribution of MGC803 cells in cell cycle (%)

| | Control | SB203580 (10 μmol/L) | DADS (30 mg/L) | DADS (30 mg/L) +SB203580 (10 μmol/L) |
|---|---|---|---|---|
| G1 | 62.5±0.9 | 62.8±1.3 | 33.7±1.2 | 54.0±1.5 |
| S | 28.2±1.2 | 27.3±0.8 | 27.9±0.8 | 24.8±1.1 |
| G2 | 9.3±0.8 | 9.9±1.1 | 39.4±1.3[a] | 21.2±0.9[a] |

[a]$P<0.05$ *vs* control.

### *Effect of reagents on cell cycle distribution of MGC803 cells*

Flow cytometry revealed that the proportion of cells in the G2/M phase after treatment with 30 mg/L DADS for 24 h was 39.4%, three times more than that in untreated cells (9.3%). SB203580 10 μmol/L alone had no effect on cell growth ($P>0.05$). But inhibition of p38 activity by SB203580 abrogated induction of G2/M arrest by DADS. MGC803 cells treated with DADS in the presence of SB203580 decreased the G2/M phase to 21.2%, compared to 39.4% by DADS alone ($P<0.05$).

### *Expression of p38, pp38, and Cdc25C after reagents treatment of MGC803 cells*

Western blotting revealed that phosphorylation of p38 was increased 3.52-fold following treatment of cells with 30 mg/L DADS for 20 min. At the same time, the total p38 amount did not change. Furthermore, DADS-induced-phosphorylation of p38 was completely inhibited by SB203580 (10 μmol/L) (Figure 1).

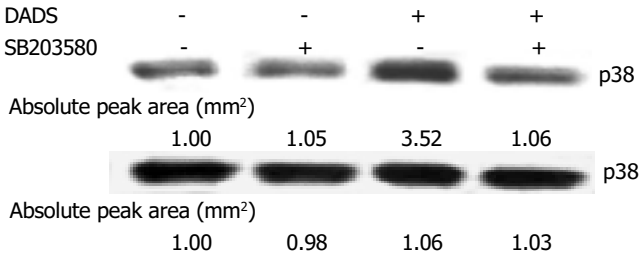


**Figure 1** Expression of p38, phosphorylation of p38 proteins in MGC803 cells following treatment of reagents for 20 min.

DADS treatment for 24 h decreased the level of Cdc25C by 68%, and pretreatment of MGC803 cells with SB203580 partially reversed the down-regulation of Cdc25C level by DADS. In contrast, SB203580 alone had no significant effect on Cdc25C expression (Figure 2).

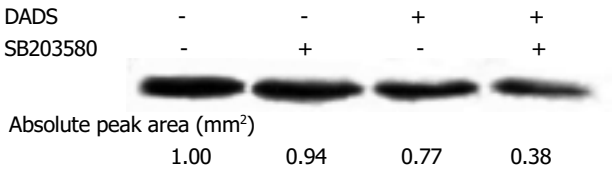


**Figure 2** Expression of Cdc25C protein in MGC803 cells following treatment of reagents for 24 h.

## DISCUSSION

Mitogen-activated protein kinase (MAPK) pathway has a central role in transducing extracelluar signals into cellular responses. p38 kinase is a member of the mitogen-activated protein kinase family that is activated by a variety of environmental stress. Rapid initiation of G2 arrest after UV radiation is mediated by p38 kinase[32]. Inhibition of p38 blocks the rapid initiation of G2 delay in both human and murine cells after ultraviolet radiation. p38 kinase is responsible for rapid initiation of the G2 delay in IME cells after the hypertonic stress created by addition of NaCl[33]. Inhibition of p38 kinase blocks the rapid initiation of this checkpoint both in an immortalized cell line (mIMCDs) and in second-passage IME cells from mouse inner medulla. Genistein-induced G2/M arrest is associated with the activated p38 mitogen-activated protein kinase[34]. Thus p38 is a critical event for initiating the G2/M checkpoint and inducing G2/M arrest. Our research showed that DADS-induced MGC803 cells

G2/M arrest and growth inhibition correlated with increased p38 phosphorylation. We used SB203580, a specific inhibitor of p38 to address the potential role of p38 kinase in the regulation of cell-cycle progression. Inhibition of p38 activity by SB203580 abolished induction of G2/M arrest by DADS. Therefore, our research data demonstrated that DADS- induced G2/M arrest of MGC803 cells involved activation of p38 MAP kinase pathways.

$p34^{cdc2}$ is the key regulator of cell-cycle progression through G2-M[35]. In particular, activation of $p34^{cdc2}$ kinase activity is required for progression from G2 to M. phosphorylation of the inhibitory residues Thr14/Tyr15 of $p34^{cdc2}$ leads to decreased kinase activity and subsequent arrest at the G2/M phase[36]. The Cdc25C protein phosphatase is a key regulator of $p34^{cdc2}$ phosphorylation status and kinase activity by dephosphorylating Thr14/Tyr15 residues[37]. *In vitro*, p38 binds and phosphorylaties Cdc25C at serine 216[32]. Phosphorylation of Cdc25C triggers cell-cycle arrest by the sequestration of Cdc25C by 14-3-3[38]. Frey *et al*. found that p38 was involved in genistein-induced G2/M arrest and down-regulation of Cdc25C expression in immortalized human mammary epithelial cell line MCF-10F[35]. Hepatitis B virus X protein (pX) is implicated in hepatocarcinogenesis by an unknown mechanism. Research data[39] showed that pX-dependent activation of p38 MAPK inactivated Cdc25C by phosphorylation of Ser216, thus initiating activation of the G2/M checkpoint, resulting in 4pX-1 cell growth retardation. These data suggest that p38 participation in down-regulation of the Cdc25C level may be an important way to impair its actions and an important event in G2/M checkpoint regulation. In the present studies, decreased Cdc25C protein phosphatase by DADS was attenuated by SB203580. Thus it indicates that regulation of Cdc25C protein expression by p38 is a critical event for G2/M arrest after treatment with DADS.

In summary, DADS-induced G2/M arrest and growth inhibition of MGC803 cells involves activation of p38 MAP kinase pathways. Decreased Cdc25C protein expression by p38 is a critical event for G2/M arrest by DADS. However, it should be noted that although p38 inhibitor SB203580 abrogated DADS-induced G2/M arrest, the reversal was not total. SB203580 could not completely abolish induction of G2/M arrest by DADS. This implies that p38 activation is not the sole prerequisite for DADS induced G2/M arrest, other mechanisms may be involved in G2/M arrest Additional studies are needed to clarify these mechanisms. A deeper understanding of the molecular mechanisms involved in the regulation of cell cycle control is very important for the development of novel anticancer strategies.

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