Growth inhibitory effect and Chk1-dependent signaling involved in G₂/M arrest on human gastric cancer cells induced by diallyl disulfide

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Abstract
Diallyl disulfide (DADS) inhibits growth and induces cell cycle G₂/M arrest in human gastric cancer MGC803 cells. In this study, 15 mg/L DADS exerted similar effects on growth and cell cycle arrest in human gastric cancer BGC823 cells. Due to the importance of cell cycle redistribution in DADS-mediated anti-carcinogenic effects, we investigated the role of checkpoint kinases (Chk1 and Chk2) during DADS-induced cell cycle arrest. We hypothesized that DADS could mediate G₂/M phase arrest through either Chk1 or Chk2 signal transduction pathways. We demonstrated that DADS induced the accumulation of phosphorylated Chk1, but not of Chk2, and that DADS down-regulated Cdc25C and cyclin B1. The expression of mRNA and total protein for Chk1 and Chk2 was unchanged. Chk1 is specifically phosphorylated by ATR (ATM-RAD3-related gene). Western blot analysis showed that phospho-ATR was activated by DADS. Taken together, these data suggest that cell cycle G₂/M arrest, which was associated with accumulation of the phosphorylated forms of Chk1, but not of Chk2, was involved in the growth inhibition induced by DADS in the human gastric cancer cell line BGC823. Furthermore, the DADS-induced G₂/M checkpoint response is mediated by Chk1 signaling through ATR/Chk1/Cdc25C/cyclin B1, and is independent of Chk2.

Key words: Diallyl disulfide; Cell cycle G₂/M arrest; Checkpoint kinase-1; Gastric cancer

Introduction
Gastric cancer is one of the leading causes of death of cancer patients and is the most common type of malignancy in China (1). Despite recent advances in surgical and chemotherapeutic procedures, the 5-year survival rate for gastric cancer patients remains very low. Hence, identifying alternative factors that may reduce the initiation and promotion of gastric cancer is important for minimizing the incidence and severity of this disease.

A high intake of garlic is associated with a protective effect against various cancers, including stomach cancer, in humans (2). Several epidemiologic studies have suggested that garlic plays an important role in the reduction of cancer-related deaths (3). Garlic oil, or more specifically its diallyl disulfide (DADS) content, has become a quite appealing anti-carcinogenic agent. This is due, in part, to its ability to induce apoptosis in vitro (4) and to inhibit the formation and growth of tumors in vivo (5,6). DADS has been shown to inhibit the in vitro and in vivo growth of breast, liver, lung, gastric, colon, and prostate cancers, as well as neuroblastoma and leukemia cell lines (5,7-14). Therefore, DADS may be a useful therapeutic tool for the prevention of environmentally induced cancers.

The antiproliferative property of DADS in cultured human colon tumor cells (HCT-116 and COLO 205) and the prostate cancer cell line (PC-3) is related to its ability to decrease the proportion of cells in the G₁ phase and to increase the proportion of cells in the G₂/M phase (12,15,16). Changes in activity may also result from a combination of the quantity and activity of specific cellular proteins. For example, hyperphosphorylation of p34(cdc2) kinase was increased by 15% following exposure of colon cells to DADS (17) and DADS also decreased Cdc25C protein expression. These findings suggest that the ability of DADS to inhibit p34(cdc2) kinase activation is due to decreased p34(cdc2)/cyclin B complex formation, as well as a shift in the p34(cdc2) hyperphosphorylation state (17). Ashra
et al. (18) demonstrated that elevated phosphorylation of checkpoint kinase 1 (Chk1), decreased phosphorylation of Chk2, and decreased levels of Cdc25C, 14-3-3, and cyclin B1 were the critical changes associated with the abrogation of G2/M checkpoint control during transformation of Syrian hamster embryo cells by Malachite green.

We have previously reported that DADS inhibited the growth of gastric cancer in vitro and in vivo (9,10), and that it induced cell cycle G2/M arrest in human gastric cancer MGC803 cells (19). A decreased Cdc25C expression plays a crucial role in G2/M arrest after treatment with DADS (19). In the current study, DADS was shown to induce phosphorylation of ATR (ATM-RAD3-related gene) and Chk, while suppressing expression of Cdc25C and cyclin B1 in the human gastric cancer cell line BGC823. These observations contribute to our understanding of the mechanisms of the anti-tumor effect of DADS in gastric cancer cells, and furthermore they indicate the potential of DADS for clinical development as a therapeutic drug for combating gastric cancer.

Material and Methods

Material

DADS (MW 146.28) and dimethyl thiazolyl tetrazolium bromide (MTT) were purchased from Fluka Chemika (Ronkonkoma, USA). The kit for bicinchoninic acid protein quantification was manufactured by Pierce (USA). The cell cycle protein test kit, which included the phospho-Chk1 (Ser345) antibody, was from Cell Signaling Technology (USA). The phospho-Chk2 (Thr68) antibody, ATR, and anti-rabbit IgG (HRP-linked) Chk1 and Chk2 antibodies were from Santa Cruz Company (USA). The Cdc25C antibody was from NeoMarkers (USA). The cyclin B1 and β-actin antibodies were from Boster Company (China). Phospho-ATR (Ser428) and ECL Lumiglo reagent were from Cell Signaling Technology.

Cell culture. The human gastric cancer cell line BGC823 was obtained from the Cell Research Institute of the Chinese Academy of Science (China). The cells were maintained in RPMI-1640 medium (Sigma, USA), supplemented with 10% heat-inactivated fetal bovine serum (FBS; Life Technologies, USA), 100 μg/mL streptomycin, and 100 U/mL penicillin G (Invitrogen, USA) in a humidified atmosphere of 95% air and 5% CO2. DADS was dissolved in dimethyl sulfoxide (DMSO), mixed with culture medium, and then added to the cells on 96-well plates at four different concentrations (5, 10, 15, and 20 mg/L). The concentration of DMSO added to the medium in all concentrations of DADS was 0.01%. The 96-well culture plate was divided into seven sections, with one section for the control, while the other sections were treated with 100-μL culture media containing 5, 10, 15, and 20 mg/L DADS. The cultures were re-incubated as described above. After 96 h, 100 μL 1 mg/mL MTT solution was added to each well, the cultures were further incubated for 4 h and 100 μL 20% SDS in 50% DMSO was added. The formed crystals were gently dissolved by slowly pipetting two to three times. A microplate reader was used to measure absorbance at 570 nm for each well. The growth inhibition rate was calculated as follows: growth inhibition = [(A570/nm of treated cells) / (A570/nm of control cells)] x 100%.

Cell cycle analysis

Cells were incubated in culture media alone, or in culture media containing 15 mg/L DADS, at 37°C for 12, 24, 36, and 48 h. 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 propidium iodide-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 and the distribution of cells in the various phases of the cell cycle was measured with a flow cytometer (Coulter EPICS-XL, Beckerman, USA). The percentage of cells in the G1, S, and G2/M phases was then calculated by the CellQuest software on the flow cytometer (Coulter EPICS-XL, Beckerman, USA).

RNA extraction and RT-PCR

The RNA from the cells obtained at each time was extracted with Trizol (Invitrogen, USA). RT was performed with an RT Kit (Promega, USA). Each milligram of total RNA was used as a template, and RT-generated cDNA encoding Chk1, Chk2, and β-actin (internal control) was amplified by PCR. Primer sequences were 5’-CTGAAGAAGCAGTC GCAATG-3’ and 5’-TTCCCACAGGACCACCATCA-3’ for Chk1; 5’-ATGTCTCGGGAGTCGGATGTTG-3’ and 5’- GCACCACTTCCAAGAGTTTGAC-3’ for Chk2; 5’-CAAGGGCACCAGCGGAAAAGA-3’ and 5’-GGATAGC ACAGCCTGGATAG-3’ for β-actin, designed by the Primer Premier 5.0 software (Premier Company, Canada) and synthesized by Shanghai Sangon Biological Technology & Services Co., Ltd. (China). The PCR products were assessed on 2% agarose gel and the concentrations of the bands on the photographs of the gels were measured with a spot density meter program (FluorChem IS-8800; Alpha Innotech, USA). The expression ratios, based on the band intensity, were then compared and analyzed. For statistical
comparison, samples were used to compare the density ratio of the objective gene to that of β-actin mRNA.

**Western blotting**

Human gastric cancer cells were cultured with or without DADS at the indicated concentrations for various periods of time, washed once with ice-cold PBS, and lysed in a buffer consisting of 20 mM Tris/HCl, pH 8.0, 137 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 10% glycerol, 100 mM NaF, and 1% Triton X-100. Protein concentration in the lysates was measured with the Protein BCA Assay Kit (Bio-Rad, USA). Thirty micrograms of protein lysate was subjected to 10% SDS-polyacrylamide gel electrophoresis and the proteins separated were transferred to polyvinylidene difluoride membranes (Millipore, USA). To block nonspecific binding, membranes were incubated at room temperature for 1 h with 5% skim milk powder, followed by a 12-h incubation at 4°C with an antiserum containing antibodies against Chk1, Chk2, ATR, Phospho-Chk1 (Ser345), Phospho-Chk2 (Thr68), Phospho-ATR (Ser428), Cdc25C, and cyclin B1. A peroxidase-conjugated secondary antibody (1:5000 dilution) and ECL Western blotting detection reagents were used to visualize the target proteins (ECL New England Biolabs, USA), which were quantified with a Bio Image Intelligent Quantifier 1-D (Version 2.2.1, Nihon-BioImage Ltd., Japan).

**Immunoprecipitation and Western blotting**

Human gastric cancer cells were cultured with or without DADS at the indicated concentrations for various periods of time, and then characterized with the Seize (R) Classic Mammalian Immunoprecipitation kit (Piece Technology, USA). The cells were carefully removed from the culture medium, washed once with PBS (0.1 M phosphate, 0.15 M NaCl, pH 7.2), harvested and lysed with the lysis buffer of the M-PER reagent. Lysates were collected, transferred to a microcentrifuge tube and centrifuged at 13,000 g for 5-10 min to separate the cell debris. The supernatants were transferred to another tube for further analysis. Purified Chk1 or Chk2 antibody was added to the sample, followed by overnight incubation at 4°C. The immune complex was added to the spin cup containing equilibrated protein G beads. Then, 190 μL elution buffer was added to the spin cup and the samples were eluted. The levels of immunoprecipitated protein were determined by Western blotting with the anti-Cdc25C antibody and the ECL detection system.

**Statistical analysis**

Data are reported as means ± SD. One-way ANOVA was used to compare difference among groups and P values of less than 0.05 were considered to be statistically significant.

**Results**

**Cell viability**

DADS inhibits the growth of BGC823 cells for 96 h, in a dose-dependent manner. The exposure of BGC823 cells to 5 mg/L DADS for 96 h inhibited growth by 34.2%/96 h, whereas exposure to 15 mg/L DADS increased the inhibition to 55.7%/96 h, as assessed by the MTT test (P < 0.05; Table 1). From these results, the IC₅₀ value of DADS for 96 h was calculated as ≈15 mg/L DADS. Hence, for further studies, we selected 15 mg/L DADS IC₅₀ to maximize the effects of dose levels so that the mechanism of action of DADS could be identified. The results indicated that the proliferation of BGC823 cells was significantly reduced by treatment with DADS for 96 h in a dose-dependent manner.

**Cell cycle arrest of BGC823 cells**

DADS inhibited the proliferation of BGC823 cells, as also shown by FACS analysis. As described previously, the inhibitory effects of DADS on proliferation are often due to cell cycle arrest (15). Consistent with its effect on cell growth inhibition, DADS induced a significant cell cycle arrest at the G₂/M phase in BGC823 cells. As shown in Table 2, approximately 48.3 (24 h) and 18.6% (24 h) of the untreated cells were in the G₀/G₁ and G₂/M phases, respectively, and 33.1% (24 h) of the cells were in the S phase. In contrast, after treatment with 15 mg/L DADS, the proportion of cells in the G₂/M phase was 58.1 (24 h) and 50.2% (36 h), or more than three times and two times, re-

| Table 1. Inhibitory effects on human gastric cancer BGC823 cells exposed to various concentrations of diallyl disulfide (DADS) for 96 h. |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| BGC823 cells    | DADS (mg/L)     |
|                 | 5   | 10  | 15  | 20  |
| Absorption value| 0.79 ± 0.049   | 0.52 ± 0.033*  | 0.48 ± 0.018*  | 0.35 ± 0.023*  | 0.24 ± 0.032*  |
| Inhibition rate(%/96 h) | 34.2 | 39.2 | 55.7 | 69.6 |

The number of BGC823 cells is reported as 2 x 10⁵/mL. The initial concentration of cells was 1 x 10⁵/mL. Data are reported as means ± SD for N = 6. *P < 0.05 vs BGC823 cells (t-test).
spectively, compared to untreated cells. Taken together, these data suggest that DADS reduces the proliferation of BGC823 cells by increasing the proportion of cells in the G2/M stage of the cell cycle.

**Effect of DADS on Chk1 and Chk2 mRNA expression in BGC823 cells**

The expression of Chk1 and Chk2 mRNA associated with the cell cycle arrest of BGC823 cells after treatment with 15 mg/L DADS for 1 or 2 days was revealed by RT-PCR. As shown in Figure 1, the expression of Chk1 and Chk2 mRNA was unchanged in treated cells compared to untreated cells.

**Expression of Chk1 and phospho-Chk1 in BGC823 cells induced by DADS**

Using antibodies specific for Chk1 phosphopeptides, we analyzed Chk1 phosphorylation at serine 345, following exposure of BGC823 cells to DADS. As illustrated in Figure 2, after stimulation with 15 mg/L DADS for 2 h, phospho-Chk1 showed a significant increase and this expression continued to increase gradually with time (P < 0.05). On the other hand, the levels of total Chk1 protein and β-actin, an inner control, did not change significantly.

**Expression of Chk2 and phospho-Chk2 induced by DADS in BGC823 cells**

Figure 3 shows the effects of DADS on the expression of phospho-Chk2 in BGC823 cells at different

<table>
<thead>
<tr>
<th>Group</th>
<th>G1 (%)</th>
<th>S (%)</th>
<th>G2/M (%)</th>
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</thead>
<tbody>
<tr>
<td>Control</td>
<td>49.1 ± 1.25</td>
<td>32.7 ± 0.83</td>
<td>18.2 ± 0.91</td>
</tr>
<tr>
<td>12 h</td>
<td>48.3 ± 1.14</td>
<td>33.1 ± 1.09</td>
<td>18.6 ± 1.82</td>
</tr>
<tr>
<td>24 h</td>
<td>50.1 ± 2.40</td>
<td>30.7 ± 1.52</td>
<td>19.2 ± 1.34</td>
</tr>
<tr>
<td>36 h</td>
<td>50.2 ± 2.21</td>
<td>29.4 ± 1.11</td>
<td>20.4 ± 1.42</td>
</tr>
<tr>
<td>48 h</td>
<td>38.4 ± 1.07</td>
<td>31.5 ± 0.31</td>
<td>30.1 ± 1.20*</td>
</tr>
<tr>
<td>12 h</td>
<td>15.1 ± 0.19</td>
<td>26.8 ± 0.23</td>
<td>58.1 ± 1.45*</td>
</tr>
<tr>
<td>24 h</td>
<td>35.6 ± 0.62</td>
<td>14.2 ± 0.09</td>
<td>50.2 ± 1.71*</td>
</tr>
<tr>
<td>36 h</td>
<td>47.7 ± 0.96</td>
<td>16.1 ± 0.18</td>
<td>36.2 ± 1.36*</td>
</tr>
</tbody>
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The concentration of BGC823 cells was 1 x 10^4/mL. Data are reported as means ± SD for N = 3. *P < 0.05 compared with BGC823 cells (t-test).

![Figure 1](image1.png)

**Figure 1.** Expression of Chk1 and Chk2 mRNA induced by diallyl disulfide (DADS). BGC823 cells (1 x 10^4/mL) were treated with 15 mg/L DADS and expression of Chk1 and Chk2 mRNA was analyzed by RT-PCR. The relative amount of each mRNA was compared to β-actin. The data are reported as means ± SD (N = 3) on the right side of the figure. Each result is the average of two determinations. Left side of the figure: Lane 1, marker; lane 2, BGC823 cells; lanes 3 and 4, BGC823 cells treated with 15 mg/L DADS for 1 or 2 days. Right side of the figure: column 1 = BGC823 cells; column 2 = BGC823 cells treated with 15 mg/L DADS for 1 day; column 3 = BGC823 cells treated with 15 mg/L DADS for 2 days. There were no significant differences (t-test).
Activation of Chk1 in G2/M arrest by diallyl disulfide

Figure 2. Activation of Chk1 phosphorylation by diallyl disulfide (DADS). BGC823 cells (1 x 10⁶/mL) were treated with 15 mg/L DADS. Expression and phosphorylation of Chk1 (P-Chk1) were analyzed by Western blot analysis. The relative amount of each protein was compared to β-actin. The data are reported as means ± SD (N = 3). Each result is the average of two determinations. *P < 0.05 vs control (t-test).

Figure 3. Activation of Chk2 phosphorylation by diallyl disulfide (DADS). BGC823 cells (1 x 10⁶/mL) were treated with 15 mg/L DADS. Expression and phosphorylation of Chk2 (P-Chk2) were analyzed by Western blot analysis. The relative amount of each protein was compared to β-actin. The data are reported as means ± SD (N = 3). Each result is the average of two determinations. *P < 0.05 vs control (t-test).

Figure 4. Expression of Cdc25C by diallyl disulfide (DADS). BGC823 cells (1 x 10⁶/mL) were treated with 15 mg/L DADS and expression of Cdc25C was analyzed by Western blot. The relative amount of each protein was compared to β-actin. The data are reported as mean ± SD (N = 3). Each result is the average of two determinations. *P < 0.05 vs control (t-test).

times. The expression of phospho-Chk2 was weak and even weaker after stimulation with DADS, but the change was not statistically significant (P > 0.05). Chk2 and β-actin, the inner control, showed no significant changes.

Expression of Cdc25C and cyclin B1, downstream molecules of Chk, induced by DADS in BGC823 cells

The results in Figure 4 show that, after 12 h, 15 mg/L DADS inhibited the expression of the cell cycle-associated phosphatase Cdc25C in BGC823 cells, which was decreased by 81% after 48 h (P < 0.05). As shown in Figure 5, the expression of cyclin B1 increased after 12 h of DADS treatment, and then decreased after 36 h, being reduced by 87.5% after 48 h (P < 0.05).

Role of ATR, as an upstream molecule of Chk, in BGC823 cells treated with DADS

ATR mediates checkpoint signaling through its downstream effect or Chk1 (20). As shown in Figure 6, treatment of BCC823 cells for 15 min to 2 h resulted in an increase in phospho-ATR expression, whereas no change was found in ATR expression.

Chk1 or Chk2 interacts with Cdc25C

The interaction of the anti-Chk1 or Chk2 antibody (Ab) with Cdc25C was assessed in immunoprecipitation experi-
ments. The Chk1 Ab increasingly precipitated Cdc25C (Figure 7A). In contrast, Chk2 Ab failed to precipitate Cdc25C in BGC823 cells (Figure 7B). Unrelated rabbit immunoglobulin (IgG) was used as a negative control.

Discussion

DADS, an oil-soluble organosulfur compound and allyl mercaptan, is formed in individuals who eat raw garlic. Moreover, DADS is the major component of cooked garlic, which is reduced to allyl mercaptan in blood (21). DADS is reported to make up about 60% of garlic oil (22), thus, indicating that it is the most appropriate compound to use in the study of the possible effects of raw and cooked garlic in humans. Studies have shown that DADS suppresses the proliferation of cancer cells such as MGC803, SW480, and HL-60-cultured cells (9,23,24). A previous study also showed that DADS inhibits the proliferation of prostate cancer cells through cell cycle arrest at the G2/M transition (12). In the present study, an apparent and dose-dependent growth inhibition characteristic was observed in the BGC823 cell line treated with DADS. At a high concentration of 15 mg/L, DADS displayed a time-dependent and steady inhibition that was not demonstrated at lower concentrations. Cell cycle arrest at the G2/M transition was shown to be involved in the growth inhibition induced by DADS in the human gastric cancer cell line.

Anticancer insights derived from cell cycle research have given rise to the idea that abrogation of the cell cycle G2 checkpoint can be viewed as a potential cancer cell-specific therapy. This idea is based on the discovery that many cancer cells have a defective G1 checkpoint that results in a dependence on the G2 checkpoint during cell replication (25,26). The cell cycle has recently become a new, more appealing target at which anti-carcinogenic agents can be directed. This represents a new mechanism for therapeutic drugs as regulators of Chk in tumor cells (27). In the present study, treatment with DADS produced cell cycle arrest in...
the G2 phase, which was associated with accumulation of the phosphorylated forms of Chk1, but not of Chk2.

Human Cdc25C is one of the central targets and regulators of the G2/M checkpoint mechanisms activated in response to DNA injury (28). Cdc25C is thought to be the major effector of the G2/M DNA damage checkpoint kinase Chk1 or/and Chk2 (29,30), which triggers the cyclin B1/CDK1 complex (30). The present study demonstrated that phospho-Chk1 upregulation, Cdc25C and cyclin B1 down-regulation in human gastric cancer cells enhanced the sensitivity to DADS, whereas phospho-Chk2 had no effect. These results have potentially vital implications for the mechanism of action of DADS and its future clinical use.

The subsequent degradation of Cdc25C in response to DADS was mediated by Chk1. Neither Chk1 nor Chk2 were directly responsible for the inhibition of DNA synthesis induced by DADS; however, Chk1 negatively regulated the entry of DADS-treated cells into mitosis. These findings suggest that DADS stimulates Chk1 to initiate a G2-M cell cycle checkpoint. Furthermore, it would appear that Chk1 acts to coordinate the cell cycle with DNA synthesis, thus preventing premature mitotic entry in DADS-treated cells. Co-immunoprecipitated Chk1 or Chk2 was detected by anti-Chk1 or anti-Chk2 immunoprecipitation followed by anti-Cdc25C immunoblotting. DADS treatment enhanced the binding activity of Chk1 with Cdc25C in BGC823 cells; however, it did not influence the binding activity of Chk2 with Cdc25C. This confirms that DADS induces G2/M arrest by the interaction of Chk1 and Cdc25C in BGC823 cells.

There are two possible scenarios explaining how DNA synthesis might be arrested. Firstly, arrest of DNA synthesis could be directly mediated by checkpoint activation. Alternatively, arrest might occur by other mechanisms that activate other checkpoints, for example, chain termination.

ATR is capable of specifically phosphorylating Chk1 (31). Another important finding of the present study was that ATR is required for maximal phosphorylation of Chk1 checkpoint proteins following DADS treatment. However, ATR only phosphorylates Chk1 (Ser345) and not Chk2. This substrate specificity may reflect the initial oxidative DNA strand breaks.

Our hypothesis is that the substrate specificity of these kinases is coordinated by the unique binding and activation of ATR with auxiliary proteins at the sites of DNA lesions. Hypothetically, the activation of these kinases toward selective substrates may be critical for activating specific DNA repair mechanisms. ATRs are very large proteins whose undefined amino-terminal domains could interact with numerous proteins involved in determining substrate specificities toward checkpoint control. Future studies should evaluate how DADS stimulates selective activation of phosphoinositide 3-kinase-related protein kinases such as ATR and DNA-dependent protein kinase. This would further clarify how these substrates regulate the cellular response to DADS.

Acknowledgments

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