We have shown previously that diallyl trisulfide (DATS), a constituent of processed garlic, inhibits proliferation of PC-3 and DU145 human prostate cancer cells by causing G2-M phase cell cycle arrest in association with inhibition of cyclin-dependent kinase 1 activity and hyperphosphorylation of Cdc25C at Ser216. Here, we report that DATS-treated PC-3 and DU145 cells are also arrested in mitosis as judged by microscopy following staining with anti-α-tubulin antibody and 4',6-diamidino-2-phenylindole and flow cytometric analysis of Ser10 phosphorylation of histone H3. The DATS treatment caused activation of checkpoint kinase 1 and checkpoint kinase 2, which are intermediaries of DNA damage checkpoints and implicated in Ser216 phosphorylation of Cdc25C. The diallyl trisulfide-induced Ser216 phosphorylation of Cdc25C as well as mitotic arrest were significantly attenuated by knockdown of checkpoint kinase 1 protein in both PC-3 and DU145 cells. On the other hand, depletion of checkpoint kinase 2 protein did not have any appreciable effect on G2 or M phase arrest or Cdc25C phosphorylation caused by diallyl trisulfide. The lack of a role of checkpoint kinase 2 in diallyl trisulfide-induced phosphorylation of Cdc25C or G2-M phase cell cycle arrest was confirmed using HCT-15 cells stably transfected with phosphorylation-deficient mutant (T68A mutant) of checkpoint kinase 2. In conclusion, the results of the present study suggest existence of a checkpoint kinase 1-dependent mechanism for diallyl trisulfide-induced mitotic arrest in human prostate cancer cells.

Epidemiological studies continue to support the premise that dietary intake of Allium vegetables such as garlic may reduce the risk of different types of malignancies including cancer of the prostate (1–5), which is the second leading cause of cancer-related deaths among men in the United States (6). Cancer protective effect of Allium vegetables is attributed to organosulfur compounds (OSCs)1 that are generated upon processing (cutting or chewing) of these vegetables (5, 7). Garlic-derived OSCs including diallyl sulfide, diallyl disulfide (DADS), and/or diallyl trisulfide (DATS) have been shown to offer significant protection against cancer in animal models induced by a variety of chemical carcinogens including dimethylnitrosamine-induced colon cancer in rodents, benzo[a]pyrene-induced fore-stomach and pulmonary carcinogenesis in mice, and azoxymethane-induced colon cancer in rats to name a few (8–17). Our own work has revealed that DATS administration suppresses growth of H-ras oncogene-transformed NIH 3T3 tumor xenografts in nude mice by inhibiting membrane association of p2111-1ras (18, 19).

Evidence is accumulating to indicate that certain naturally occurring OSC analogues can suppress proliferation of cancer cells in culture by causing cell cycle arrest and apoptosis (20–31). For example, DADS inhibited proliferation of HCT-15 human colon cancer cells by causing G2-M phase cell cycle arrest and apoptosis induction (20–22). In HCT-15 cells, accumulation of G2-M phase cells upon treatment with DADS was accompanied by an increase in cyclin B1 protein level, reduction in complex formation between cyclin-dependent kinase 1 (Cdk1) and cyclin B1, and hyperphosphorylation of Cdk1 (21, 22). The DADS-induced apoptosis in HCT-15 cells correlated positively with an increase in the intracellular free calcium level (20). In MDA-MB-231 human breast cancer cell line, the DADS-induced apoptosis was associated with induction of Bax protein, down-regulation of Bel-XL, and activation of caspase-3 (23). Recently, we determined the effects of OSC analogues with varying oligosulfide chain length (diallyl sulfide, DADS, and DATS) on proliferation of human prostate cancer cells (29). We found that DATS is a much more potent apoptosis inducer than either diallyl sulfide or DADS, indicating that even a subtle change in OSC structure (e.g. oligosulfide chain length) could have a significant impact on its biological activity (29). We demonstrated further that the DATS-induced cell death in prostate cancer cells was due to c-Jun N-terminal kinase and extracellular signal-regulated kinase-mediated phosphorylation of Bel-2 (29). Although significant progress has been made toward our understanding of the signal transduction pathways leading to OSC-mediated apoptosis (reviewed in Ref. 30), the mechanism by which these highly promising cancer chemopreventive agents block cell cycle progression remains elusive.

We have shown previously that DATS-induced G2-M phase cell cycle arrest in human prostate cancer cells is associated with reactive oxygen species-dependent destruction as well as hyperphosphorylation of Cdc25C at Ser216 (31). In the present study, we extended these observations, and determined the mechanism and functional significance of Cdc25C hyperphosphorylation in DATS-induced G2-M phase cell cycle arrest. Here, we demonstrate that DATS-treated PC-3 and DU145 cells are also arrested

serum albumin; DAPI, 4',6-diamidino-2-phenylindole; siRNA, small interfering RNA.

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1 The abbreviations used are: OSC, organosulfur compound; DADS, diallyl disulfide; DATS, diallyl trisulfide; Cdk1, cyclin-dependent kinase 1; Cdc25C, cell division cycle 25C; Chk1 and -2, checkpoint kinase 1 and 2, respectively; PBS, phosphate-buffered saline; BSA, bovine

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In M (mitotic) phase in association with activation of checkpoint kinase 1 (Chk1) and checkpoint kinase 2 (Chk2). Furthermore, the present study indicates that the DATS-induced hyperphosphorylation of Cdc25C and mitotic arrest are significantly attenuated by siRNA-based knockdown of Chk1 protein. On the other hand, Chk2 protein knockdown does not affect DATS-induced G2 or mitotic block. In conclusion, the present study indicates that the DATS-induced mitotic arrest in human prostate cancer cells is regulated by Chk1, which is an intermediary of DNA damage checkpoint (32–34).

MATERIALS AND METHODS

Reagents—DATS (purity ~98%) was purchased from LKT Laboratories (St. Paul, MN). Oligofectamine, G418, tissue culture media, penicillin/streptomycin antibiotic mixture, and fetal bovine serum were from Invitrogen; propidium iodide and phosphatase inhibitors were from Sigma; RNase A was from Promega (Madison, WI); and protease inhibitor mixture was from BD Pharmingen (San Diego, CA). The antibodies against Cdc25C (sc-13138), phospho-(Ser161)-Cdc25C (sc-12354), ATR (ataxia-telangiectasia mutated (ATM) and Rad3-related protein kinase; sc-1887), Cdc25A (sc-7157), Chk2 (sc-9064), phospho-(Ser79)-Chk2 (sc-16297), and cyclin H1 (sc-245) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The antibodies against Chk1 (catalog number 2345) and phospho-(Ser177)-Chk1 (catalog number 12344) were from Cell Signaling Technology (Beverly, MA). The antibodies against phospho-(Ser10)-histone H3 (catalog number 06-570) and phospho-(Ser139)-H2AX (catalog number 05-638) were from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-actin antibody was from Oncogene Research Products (San Diego, CA), anti-phospho-(Ser1981)-ATM antibody (catalog number 600-401-400) was from Rockland (Gilbertsville, PA), and anti-p53 antibody (catalog number OP43) was from BioMedical. The fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (catalog number 111-095-003) was from Jackson Immunoresearch Laboratories (West Grove, PA).

Cell Lines and Cell Culture—Monolayer cultures of PC-3 cells were maintained in F-12K Nutrient Mixture (Kaighn’s modification) supplemented with L-glutamine, antibiotics, and 7% (v/v) non-heat-inactivated fetal bovine serum and antibiotics. Monolayer cultures of DU145 cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, antibiotics, and fetal bovine serum, and antibiotics. The HCT-15 cells stably transfected with HA-tagged wild type Chk2 or HA-tagged T68A mutant of Chk2 were maintained in F-12K Nutrient Mixture (Kaighn’s modification) supplemented with 7% (v/v) non-heat-inactivated fetal bovine serum and antibiotics. Monolayer cultures of DU145 cells were maintained in F-12K Nutrient Mixture (Kaighn’s modification) supplemented with 7% (v/v) non-heat-inactivated fetal bovine serum and antibiotics. Monolayer cultures of DU145 cells were maintained in Eagle’s minimum essential medium supplemented with l-glutamine (Cellgro, Herndon, VA), 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 10% (v/v) fetal bovine serum, and antibiotics. The LNCaP cultures were maintained in RPMI 1640 supplemented with l-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 0.2% glucose, 10% fetal bovine serum, and antibiotics. The HCT-15 cells stably transfected with HA-tagged wild type Chk2 or HA-tagged T68A mutant of Chk2 were kindly provided by Dr. Junjie Chen (Mayo Clinic, Rochester, MN) (35, 36) and were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, antibiotics, and 200 μg G418/ml. Each cell line was maintained at 37 °C in an atmosphere of 95% air and 5% CO2.

Immunofluorescence Microscopy—PC-3 cells (2 × 10⁵) were grown on coverslips and allowed to attach overnight. The cells were then exposed to Me2SO or 40 μM DATS for specified time periods at 37 °C, washed with phosphate-buffered saline (PBS), and fixed at 4 °C overnight with 2% paraformaldehyde. Stock solution of DATS was prepared in Me2SO, and an equal volume of Me2SO was added to controls. The cells were permeabilized with 0.1% Triton X-100 for 15 min at room temperature, washed with PBS, and incubated with normal goat serum (1:20 dilution) for 45 min at room temperature. After washing with BSA buffer, the cells were treated with anti-α-tubulin (1:4000 dilution) or anti-phospho-(Ser1981)-H2AX (2 μg/ml) antibody for 1 h at room temperature. Cells were then washed with BSA buffer and incubated with 1 μg/ml Alexa Fluor 568-conjugated goat anti-mouse antibody (catalog number A-11004; Molecular Probes, Inc., Eugene, OR) for 1 h at room temperature followed by counterstaining with 10 ng/ml 4′,6-diamidino-2-phenylindole (DAPI). Slides were mounted and examined under a Leica fluorescence microscope at ×100 (objective lenses) magnification. The mitotic figures with condensed chromatin were counted. A total of 300 cells were scored in two separate fields of each sample.

Flow Cytometric Analysis of Phospho-(Ser10)-histone H3—The effect of DATS treatment on Ser10 phosphorylation of histone H3, a sensitive marker for mitotic cells (37), was determined by flow cytometry as described by Widrow and Laird (38) with some modifications. Briefly,
the cells were treated with DATS as described above, fixed in 70% ethanol at 4 °C, suspended in 1 ml of 0.25% Triton X-100 in PBS, and incubated on ice for 15 min. The cells were collected, suspended in 100 µl of PBS containing 1% BSA and 0.75 µg of a polyclonal anti-phospho-(Ser19)-histone H3 antibody, and incubated at 4 °C overnight. The cells were then rinsed with PBS containing 1% BSA and incubated in the dark with fluorescein isothiocyanate-conjugated goat anti-rabbit antibody (1:50 dilution in PBS containing 1% BSA) for 30 min at room temperature. The cells were washed with PBS containing 1% BSA and treated with propidium iodide and RNase A for 30 min as described previously. Cellular fluorescence was measured using a Coulter Epics XL flow cytometer.

Immunoblotting—Cells were treated with DATS as described above and lysed using a solution containing 50 mM Tris (pH 8), 1% Triton X-100, 0.1% SDS, 150 mM NaCl, protease inhibitor mixture, 2 mM Na3VO4, 2 mM EGTA, 12 mM β-glycerophosphate, and 10 mM NaF. The lysate was clarified by centrifugation at 14,000 rpm for 15 min. Lysate proteins were resolved by SDS-PAGE and transferred onto polyvinylidene difluoride membrane. The membrane was treated with a solution containing 10 mM Tris (pH 7.4), 150 mM NaCl, 0.05% Tween 20, and 5–10% nonfat dry milk and incubated with the desired primary antibody for 2 h at room temperature. The membrane was treated with appropriate secondary antibody for 1 h at room temperature. The immunoreactive bands were visualized by the enhanced chemiluminescence method. The blots were stripped and reprobed with anti-actin antibody to normalize for differences in protein loading. Change in protein level was determined by densitometric scanning of the immunoreactive bands and corrected for actin loading control.

Analysis of Cell Cycle Distribution—Cell cycle distribution in control (Me2SO-treated) and DATS-treated cells was determined by flow cytometry as described by us previously (39). Briefly, exponentially growing cells were exposed to Me2SO (control) or DATS for the desired time periods. Both floating and adherent cells were collected, washed with PBS, fixed with 70% ethanol, stained with propidium iodide, and analyzed using a Coulter Epics XL flow cytometer as described by us previously (39).

siRNA Transfection—RNA interference of Chk1 and Chk2 was performed using annealed double-stranded oligonucleotides (Dharmacon, Lafayette, CO). For PC-3 cells, the coding strands for Chk1 and Chk2 siRNA were CGUGCGCGUAGACUGUCCAdTdT and GAAACCUGAGGACAAAGACdTdT, respectively. Mock-treated cells as well as cells transfected with a nonspecific control siRNA (ACUUCAGCGCAUUCGUGCUCUU (Dharmacon; PC-3 cells) or UUCUCCGGAAGGCUGACGUGCGdTdT (Qiagen; DU145 cells)) were used as controls. The coding strand for the Chk1 siRNA used for DU145 cells was GAAGCAUGCCGCAUGAAGAdTdT (Dharmacon). For transfection, cells were plated in 6-well plates and transfected at 30% confluence with siRNA duplexes (200 nM) using Oligofectamine according to the manufacturer’s recommendations. After 24 h, cells were treated with DATS or Me2SO (control) for 8 h. Both floating and adherent cells were collected, washed with PBS, and processed for cell cycle analysis, determination of histone H3 phosphorylation, or immunoblotting as described above.

Statistical Analysis—Paired t test or one-way analysis of variance (followed by Bonferroni’s or Tukey’s multiple comparison test) was used to test the significance of differences in measured variables between control and treated groups.

RESULTS

DATS Treatment Caused Mitotic Arrest in PC-3 Cells—We have shown previously that DATS-treated PC-3 and DU145 cells, but not a normal prostate epithelial cell line (PrEC), are arrested in the G2-M phase of the cell cycle in a concentration- and time-dependent manner (31). The DATS-mediated G2-M phase cell cycle arrest in prostate cancer cells is associated with generation of reactive oxygen species, inhibition of Cdk1-cyclin B1 kinase activity, and accumulation of cyclin B1 protein (31). In the present study, we extended these findings to gain further insights into the mechanism by which DATS inhibits cell cycle progression. In cycling cells, cyclin B1 protein level increases abruptly as the cells acquire 4N DNA content, peaks during the metaphase/anaphase transition, and declines precipitously upon completion of mitosis (40–42). Degradation of cyclin B1 is essential for mitotic exit (40–42). Because DATS treatment caused accumulation of cyclin B1 protein (31), we hypothesized that DATS-treated PC-3 cells might be unable to exit mitosis. We explored this possibility by immunofluorescence microscopy following staining of control and DATS-treated PC-3 cells with anti-α-tubulin antibody and DAPI. As shown in Fig. 1A (panels a–c), a large fraction of control cells exhibited an intact microtubule network. On the other hand, the α-tubulin staining in DATS-treated PC-3 cells was restricted to small areas on the periphery of the nucleus. More-
over, the DAPI staining revealed an abundance of mitotic figures in a prometaphase and metaphase-like state with condensed chromatin in DATS-treated PC-3 cultures (identified by an arrow in panels d and e of Fig. 1A), which were less frequent in vehicle-treated controls (compare panels a–c in Fig. 1A for controls). The mitotic figures with condensed chromatin were scored in cultures of control and DATS-treated PC-3 cells, and the results are summarized in Fig. 1B. The percentage of mitotic figures was ~4-fold higher in DATS treated PC-3 cultures compared with vehicle-treated controls (Fig. 1B). Interestingly, DATS-treated PC-3 cultures also exhibited multinucleated cells (identified by an arrowhead in panel f of Fig. 1A), which were rarely seen in control cultures.

The DATS-induced mitotic arrest was confirmed by flow cytometric analysis of Ser\(^{10}\) phosphorylation of histone H3, which has emerged as a sensitive marker for mitotic cells (37). The Ser\(^{10}\) phosphorylation of histone H3 begins in prophase, peaks in metaphase, and declines during anaphase (43, 44). Agents initiating premature chromosome condensation have been shown to increase Ser\(^{10}\) phosphorylation of histone H3 (45, 46). As can be seen in Fig. 1C, the Ser\(^{10}\) phosphorylation of histone H3 was increased by about 5-fold upon treatment of PC-3 cells with DATS compared with control. Collectively, these results indicated that DATS-treated PC-3 cells were arrested in mitosis.

**DATS Treatment Promoted Phosphorylation of Chk1 at Ser\(^{317}\) and Chk2 at Thr\(^{68}\) in PC-3 Cells**—We have shown previously that the DATS-induced G\(_{1}\)-M phase cell cycle arrest in PC-3 cells is associated with a marked increase in Ser\(^{216}\) phosphorylation of Cdc25C (31), which together with other Cdc25 family members plays an important role in activation of Cdk1-cyclin B1 kinase (47–49). Phosphorylation of Cdc25C at Ser216 family members plays an important role in activation of Cdk1-phosphorylation of Cdc25C (31), which together with other Cdc25 kinases. As can be seen in Fig. 2A, the level of Chk1 protein decreased in a time- and dose-dependent manner upon treatment of PC-3 cells with DATS (up to 70% reduction in DATS-treated PC-3 cells at the 24-h time point compared with control). At the same time, DATS treatment caused an increase in Ser\(^{345}\) phosphorylation of Chk1, which was negligible in control cells. The DATS-mediated Ser\(^{345}\) phosphorylation of Chk1 was evident as early as 1 h after treatment with 40 \(\mu\)M DATS (Fig. 2A). In dose-response studies, Ser\(^{345}\) phosphorylation of Chk1 was observed following a 1-h treatment of PC-3 cells with 10, 20, or 40 \(\mu\)M DATS (Fig. 2A). We also determined the effect of DATS treatment on Ser\(^{345}\) phosphorylation of Chk1 using antibodies from two commercial sources (catalog number 2341, Cell Signaling; catalog number sc-17922, Santa Cruz Biotechnology). However, we were unable to detect Ser\(^{345}\)-phosphorylated Chk1 protein in control as well as in DATS-treated PC-3 cells. The DATS-mediated Ser\(^{345}\) phosphorylation of Chk1 led to an increase in the level of Ser\(^{345}\)-phosphorylated Chk1. These results are similar to those of Gatei et al. (54), who observed phosphorylation of Chk1 at Ser\(^{345}\) but not at Ser\(^{345}\) in response to ionizing radiation exposure in lymphoblastoid and fibroblast lines. Thus, it seems reasonable to conclude that DATS treatment causes phosphorylation of Chk1 at Ser\(^{345}\) but not at Ser\(^{345}\) in PC-3 cells (Fig. 2A).

Activating phosphorylations of Chk1 and Chk2 are mainly caused by ATR and ATM, which belong to a superfamily of rather large protein kinases bearing a phosphatidylinositol 3-kinase signature at their carboxyl terminus (33, 34, 51–53, 55, 56). It has been reported that cellular irradiation induces a rapid intermolecular autophosphorylation of Ser\(^{1981}\) to initiate cellular ATM kinase activity (57). To assess if DATS-mediated Thr\(^{68}\) phosphorylation of Chk2 was associated with ATM activation, we determined the effect of DATS treatment on Ser\(^{1981}\) phosphorylation of ATM by immunoblotting, and the results

| Panel A | DATS-induced hyperphosphorylation of Cdc25C and Cdk1 in PC-3 cells is mediated by Chk1. A, immunoblotting for total Chk1, phospho-(Ser\(^{216}\))-Cdc25C, phospho-(Tyr\(^{15}\))-Cdk1, and cyclin B1 using lysates from PC-3 cells transiently transfected with a Chk1 specific siRNA and control transfectants (mock- and control siRNA-transfected) following an 8-h treatment with Me\(_{2}\)SO (control) or 40 \(\mu\)M DATS. B, immunoblotting for total Chk2, phospho-(Ser\(^{345}\))-Cdc25C, phospho-(Tyr\(^{15}\))-Cdk1, and cyclin B1 using lysates from PC-3 cells transfected with a Chk2-specific siRNA and control transfectants (mock- and control siRNA-transfected) following an 8-h treatment with Me\(_{2}\)SO or 40 \(\mu\)M DATS. The blots were stripped and reprobed with anti-actin antibody to normalize for differences in protein loading. Similar results were observed in replicate experiments.

![Figure 4](http://www.jbc.org/)

**Fig. 4.** DATS-induced hyperphosphorylation of Cdc25C and Cdk1 in PC-3 cells is mediated by Chk1. A, immunoblotting for total Chk1, phospho-(Ser\(^{216}\))-Cdc25C, phospho-(Tyr\(^{15}\))-Cdk1, and cyclin B1 using lysates from PC-3 cells transiently transfected with a Chk1 specific siRNA and control transfectants (mock- and control siRNA-transfected) following an 8-h treatment with Me\(_{2}\)SO (control) or 40 \(\mu\)M DATS. B, immunoblotting for total Chk2, phospho-(Ser\(^{345}\))-Cdc25C, phospho-(Tyr\(^{15}\))-Cdk1, and cyclin B1 using lysates from PC-3 cells transfected with a Chk2-specific siRNA and control transfectants (mock- and control siRNA-transfected) following an 8-h treatment with Me\(_{2}\)SO or 40 \(\mu\)M DATS. The blots were stripped and reprobed with anti-actin antibody to normalize for differences in protein loading. Similar results were observed in replicate experiments.
are shown in Fig. 2C. An increase in Ser\(^{1981}\) phosphorylation of ATM in DATS-treated PC-3 cells was evident as early as 1 h after treatment. The DATS-mediated phosphorylation of ATM increased gradually with increasing exposure time and was maintained for the duration of the experiment (8 h after DATS treatment). The DATS treatment did not affect ATR protein level (Fig. 2C). The Chk1 and Chk2 kinases have also been shown to phosphorylate Cdc25A and consequently regulate its stability (58, 59). The Cdc25A isoform controls the activation of Cdk1 and Cdk2 and is implicated in regulation of G, intra-S, and G-M checkpoints (58, 60, 61). The Chk1/Chk2-dependent phosphorylation of Cdc25A leads to its proteasome-mediated degradation and cell cycle arrest in response to different stimuli, including camptothecin and doxorubicin and ionizing radiation (48, 58, 59). We therefore examined the effect of DATS treatment on Cdc25A protein level by immunoblotting to determine whether the cell cycle arrest in our model was associated with altered stability of Cdc25A.

**DATS Caused Ser\(^{139}\) Phosphorylation of Histone H2A.X**—Recent studies from our laboratory have revealed that DATS-induced G-M phase cell cycle arrest in PC-3 and DU145 cells is associated with reactive oxygen species generation (31). The reactive oxygen species can either directly cause DNA damage or oxidize nucleotides that can be converted to double strand breaks during DNA replication (62, 63). The Ser\(^{139}\) phosphorylation of histone H2A.X has emerged as a sensitive marker for the presence of DNA double strand breaks (64, 65). Histone H2A.X specifically controls the recruitment of DNA repair proteins to the sites of DNA damage (66) and is phosphorylated by ATR in response to genotoxic stresses including UV and replication inhibitor hydroxyurea (65). To assess whether activation of Chk1 and Chk2 in our model was linked to DNA damage, we determined the effect of DATS treatment on Ser\(^{139}\) phosphorylation of H2A.X by microscopy, and the results are shown in Fig. 3. Etoposide, a topoisomerase II poison, was included as a positive control. As can be seen in Fig. 3, similar to etoposide, DATS treatment caused Ser\(^{139}\) phosphorylation of histone H2A.X, which was barely detectable in vehicle-treated control PC-3 cells (Fig. 3). Collectively, these results suggest that DATS treatment may cause oxidative DNA damage to activate ATR-Chk1/ATM-Chk2 in PC-3 cells.

**Role of Chk1 and Chk2 in DATS-induced Phosphorylation of Cdc25C**—Next, we raised the question of whether DATS-induced Ser\(^{216}\) phosphorylation of Cdc25C (31) was mediated by Chk1 and/or Chk2. We addressed this question by determining the effect of Chk1 and Chk2 protein knockdown on DATS-induced phosphorylation of Cdc25C. As can be seen in Fig. 4A, transient transfection of PC-3 cells with Chk1-specific siRNA duplexes caused about 70–80% depletion in the level of Chk1 protein. Similar to untransfected PC-3 cells (31), the DATS-induced Ser\(^{216}\) phosphorylation of Cdc25C was observed in mock and control siRNA-transfected PC-3 cells (−2-fold increase over Me\(_2\)SO control). On the other hand, DATS-induced Ser\(^{216}\) phosphorylation of Cdc25C was nearly fully blocked in Chk1 siRNA-transfected cells. The Chk1 protein knockdown also attenuated DATS-induced accumulation of Tyr\(^{15}\)-phosphorylated (inactive) Cdk1 (Fig. 4A). Moreover, the DATS-mediated accumulation of cyclin B1 was relatively more pronounced in mock-treated and control siRNA-transfected PC-3 cells (between 1.7- and 2.6-fold increase in DATS-treated cells compared with corresponding Me\(_2\)SO-treated controls) than in
Chk1-depleted PC-3 cells (Fig. 4A).

We also determined the effect of Chk2 protein knockdown on DATS-induced phosphorylation of Cdc25C, and the results are summarized in Fig. 4B. Transient transfection with siRNA targeted to Chk2 suppressed its protein level by about 60–70%) in comparison with mock-treated and control siRNA-transfected PC-3 cells. Similar to untransfected PC-3 cells (31), DATS treatment caused an increase in the levels of Ser216-phosphorylated Cdc25C (1.7–2.5-fold over control) and Tyr15-phosphorylated Cdk1 (1.5–2-fold over control) in mock-treated and control siRNA-transfected PC-3 cells. The level of cyclin B1 was 2–3-fold higher in DATS-treated mock and control siRNA-transfected cells than in corresponding Me2SO-treated controls. Increased phosphorylation of Cdc25C and Cdk1 as well as accumulation of cyclin B1 protein upon treatment with DATS was also observed in cells transfected with Chk2-specific siRNA duplexes (Fig. 4B). Collectively, these results indicated that the DATS-induced hyperphosphorylation of Cdc25C in PC-3 cells was mediated by Chk1 but not Chk2.

**Chk1 Protein Knockdown Attenuated DATS-induced Mitotic Arrest**—Because Chk1 protein knockdown inhibited DATS-induced hyperphosphorylation of Cdc25C and Cdk1 (Fig. 4A), we proceeded to test whether DATS-induced cell cycle arrest in our model was dependent on Chk1 protein. To test this possibility, we compared cell cycle distribution in controls (mock-treated and control siRNA-transfected) and Chk1-depleted PC-3 cells following an 8-h treatment with 40 \( \mu \)M DATS. Representative histograms for cell cycle distribution in mock control, control siRNA-transfected or Chk1 siRNA-transfected PC-3 cells following an 8-h treatment with Me2SO (control) or 40 \( \mu \)M DATS are shown in Fig. 5A. Data on the effect of Chk1 protein knockdown on DATS-mediated G2-M phase cell cycle arrest are summarized in Fig. 5B. The DATS-induced accumulation of G2-M phase cells with 4N DNA content was observed not only in controls (mock-treated and control siRNA-transfected PC-3 cells) but also in PC-3 cells transfected with Chk1-specific siRNA. The DATS-induced accumulation of G2-M phase cells was significantly reduced in Chk1 siRNA-transfected cells (Fig. 5B).
silencing Chk1 protein by siRNA duplexes (Fig. 5B). The Chk1 protein depletion (70–80%) in Chk1 siRNA-transfected cells was confirmed by immunoblotting in each transfection experiment. These results indicated that although Chk1 protein depletion inhibited DATS-induced hyperphosphorylation of Cdc25C (Fig. 4A), the G2–M phase cycle arrest caused by DATS was not abrogated by Chk1 knockdown. Next, we addressed the question of whether Chk1 protein knockdown affected DATS-induced mitotic arrest. Initially, we examined the effect of DATS treatment on Ser10 phosphorylation of histone H3 using control (mock-treated and control siRNA-transfected) and Chk1 siRNA-transfected cells. As can be seen in Fig. 6A, similar to untransfected PC-3 cells (Fig. 1C), DATS treatment (40 μM, 8 h) caused an approximately 3–4-fold increase in the level of phospho-(Ser10)-histone H3 in mock-treated and control siRNA-transfected PC-3 cells compared with corresponding Me2SO-treated controls (Fig. 6A). On the other hand, a similar DATS treatment in Chk1 siRNA-transfected PC-3 cells resulted in an increase of only about 2-fold in the level of phospho-(Ser10)-histone H3 compared with vehicle-treated control (Fig. 6A). These results indicated that the DATS-induced mitotic arrest was markedly abrogated by depletion of the Chk1 protein. In agreement with these results, the DATS-induced accumulation of mitotic figures, as judged by immunofluorescence microscopy following staining with anti-α-tubulin antibody and DAPI, was much less pronounced in PC-3 cells transfected with Chk1 siRNA than in control siRNA-transfected cells (Fig. 6B). Collectively, these results indicated that depletion of Chk1 protein level in PC-3 cells markedly reduced DATS-mediated accumulation of mitotic figures.

**Chk1 Protein Knockdown Inhibited DATS-induced Accumulation of Multinucleated Cells**—We also determined the effect of Chk1 protein knockdown on the DATS-induced appearance of multinucleated cells, and the results are summarized in Fig. 6C. The percentage of multinucleated cells on treatment with DATS (40 μM, 8 h) was increased by about 3-fold in PC-3 cells transfected with control siRNA. On the other hand, a similar DATS treatment in Chk1 siRNA-transfected cells resulted in only about a 50% increase in percentage of multinucleated cells compared with Me2SO-treated control (Fig. 6C). These results indicated that the DATS-induced accumulation of multinucleated cells was also regulated by Chk1.

**Chk2 Was Disposable for DATS-induced G2-M Phase Cell Cycle Arrest in PC-3 Cells**—Next, we determined the effect of Chk2 protein knockdown on DATS-induced accumulation of G2-M phase cells, and the results are summarized in Fig. 7A. Accumulation of cells with G2-M phase DNA content upon treatment with DATS (40 μM, 8 h) was observed not only in controls (mock-treated and control siRNA-transfected cells) but also in cells transfected with Chk2-targeted siRNA duplexes (Fig. 7A). Furthermore, as can be seen in Fig. 7B, the DATS-mediated increase in Ser10 phosphorylation of histone H3 was observed in controls (mock-treated and control siRNA-transfected PC-3 cells) as well as in Chk2-depleted PC-3 cells (Fig. 7B). Collectively, these results indicated that Chk2 protein was dispensable for DATS-induced G2 and M phase arrest.

Lack of Chk2 involvement in DATS-induced cell cycle arrest was confirmed using HCT-15 cells stably transfected with wild type Chk2 and phosphorylation-deficient mutant (T68A) of Chk2. The DATS treatment (40 μM, 8 h) caused an increase in G2-M fraction not only in cells expressing wild type Chk2 but also in cells stably transfected with the T68A mutant of Chk2 (results not shown).

**Chk1 Dependence of DATS-induced Mitotic Arrest Was Not Restricted to PC-3 Cells**—Next, we raised the question of whether the Chk1 dependence of DATS-induced M phase cell cycle arrest was restricted to the PC-3 cell line due to its unique characteristics. We addressed this issue by determining the effect of Chk1 protein knockdown on DATS-induced hyperphosphorylation of Cdc25C, G2 and M phase cell cycle arrest using DU145 prostate adenocarcinoma cells. As can be seen in Fig. 8A (top panel), transient transfection of DU145 cells with Chk1-specific siRNA duplexes caused about 70–80% depletion of Chk1 protein level. Furthermore, similar to PC-3 cells, DATS treatment (40 μM, 8 h) caused a marked increase in phosphorylation of Chk1 (Ser317), Cdc25C (Ser216), and Cdk1 (Tyr15) in DU145 cells transfected with a control siRNA (Fig. 8A). On the other hand, the DATS-induced hyperphosphorylation of Chk1, Cdc25C, and Cdk1 was either fully abrogated (Chk1 and Cdc25C) or significantly inhibited (Cdk1) in Chk1-depleted DU145 cells. These results indicated that Chk1 dependence of
DATS-induced phosphorylation of Cdc25C and Cdk1 is not unique to the PC-3 cell line.

Fig. 8 summarizes the results of studies designed to determine the effect of Chk1 protein knockdown on DATS-induced accumulation of G2-M phase cells using DU145. In control siRNA-transfected DU145 cells, an 8-hr treatment with 40 μM DATS led to an approximate 2.2-fold enrichment of G2-M fraction compared with Me2SO-treated control. A similar DATS treatment in Chk1-depleted DU145 cells also resulted in an about 2-fold increase in G2-M fraction compared with Me2SO-treated control. These results indicated that, similar to PC-3 cells (Fig. 5), Chk1 protein did not affect DATS-induced G2-M phase cell cycle arrest in DU145 (Fig. 8B). On the other hand, the DATS-induced accumulation of mitotic figures, as judged by immunofluorescence microscopy following staining with anti-α-tubulin and DAPI, was reduced significantly in DU145 cells transfected with Chk1-specific siRNA duplexes compared with control siRNA-transfected DU145 cells. For example, the control siRNA-transfected DU145 cells treated for 8 h with 40 μM DATS exhibited an approximately 5.6-fold increase in the percentage of mitotic figures compared with corresponding vehicle-treated control, which was reduced statistically significantly in Chk1-depleted DU145 cells (about 52% reduction compared with control siRNA-transfected DU145 cells) (Fig. 8C). These results indicate that, similar to PC-3, Chk1 plays an important role in DATS-induced mitotic arrest in DU145 cells.

Effect of DATS Treatment on p53 Protein Stability and Cell Cycle Distribution in LNCaP Cells—Recent studies have suggested that p53 tumor suppressor protein, which is a target of ATM/Chk2/ATR/Chk1, can also regulate G2-M transition in control. These results indicated that, similar to PC-3 cells (Fig. 5), Chk1 protein did not affect DATS-induced G2-M phase cell cycle arrest in DU145 (Fig. 8B). On the other hand, the DATS-induced accumulation of mitotic figures, as judged by immunofluorescence microscopy following staining with anti-α-tubulin and DAPI, was reduced significantly in DU145 cells transfected with Chk1-specific siRNA duplexes compared with control siRNA-transfected DU145 cells. For example, the control siRNA-transfected DU145 cells treated for 8 h with 40 μM DATS exhibited an approximately 5.6-fold increase in the percentage of mitotic figures compared with corresponding vehicle-treated control, which was reduced statistically significantly in Chk1-depleted DU145 cells (about 52% reduction compared with control siRNA-transfected DU145 cells) (Fig. 8C). These results indicate that, similar to PC-3, Chk1 plays an important role in DATS-induced mitotic arrest in DU145 cells.

Fig. 9. DATS-induced G2-M phase cell cycle arrest in LNCaP cells is associated with stabilization of p53. A, immunoblotting for p53 using lysates from LNCaP cells treated with 40 μM DATS for the indicated time periods. The blot was stripped and reprobed with anti-actin antibody to normalize for differences in protein loading. Shown are the percentages of G2-M phase cells (B) and mitotic cells (C) in LNCaP cultures treated with Me2SO or 40 μM DATS for the indicated time periods. Data are mean ± S.E. (n = 3). *, significantly different between control and DATS-treated groups by paired t test (p < 0.05).
The mechanism by which p53 activates a G2-M checkpoint involves inhibition of Cdk1 by regulating p53 transcriptional targets, including Gadd45, p21, and 14-3-3σ (67). The transcriptional activity and stability of p53 is regulated by its phosphorylation at multiple sites (reviewed in Ref. 68). For example, the ATM/Chk2-mediated Ser15 phosphorylation of p53 leads to its stabilization due to reduced interaction with Mdm2 (69, 70). Since PC-3 and DU145 cells lack wild type p53 (71), it is reasonable to conclude that the DATS-induced cell cycle arrest, at least in human prostate cancer cells, is independent of p53. Nonetheless, it was of interest to us to determine whether the presence of p53 protein influences DATS-induced cell cycle arrest. We addressed this question by determining the effect of DATS treatment on p53 protein level and cell cycle distribution using LNCaP cells, which express wild type p53 (71). As can be seen in Fig. 9A, treatment of LNCaP cells with 40 μM DATS resulted in an increase in the level of p53 protein, especially at the 4 and 8 h time points. An increase in Ser15 phosphorylation of p53 was also observed following a 4- or 8-h treatment of LNCaP cells with DATS (results not shown). At the same time, similar to PC-3 and DU145 cells, DATS treatment (40 μM, 8 or 16 h) also resulted in a statistically significant increase in G2-M fraction in LNCaP cultures at both the 8 and 16 h time points (about 1.6- and 1.8-fold increase, respectively, compared with corresponding vehicle-treated controls) (Fig. 9B). Moreover, DATS treatment caused an increase in mitotic figures in LNCaP cells as well (Fig. 9C). Collectively, these results argued against a role of p53 in DATS-induced cell cycle arrest, at least in human prostate cancer cells.

**DISCUSSION**

We have shown previously that the antiproliferative effect of DATS against PC-3 and DU145 cells correlates with inhibition of G2-M progression, whereas a normal prostate epithelial cell line is highly resistant to growth inhibition and cell cycle arrest by DATS even at concentrations that are highly cytotoxic to the prostate cancer cells (31). Our previous studies also offered novel insights into the mechanism by which DATS, a highly promising cancer chemopreventive agent present in processed garlic, may inhibit cell cycle progression. We found that the DATS-induced cell cycle arrest in prostate cancer cells is associated with accumulation of cyclin B1 (31). In cycling cells, the synthesis of B type cyclins increases abruptly during late S to early G2 peaks during the metaphase/anaphase transition, and declines precipitously upon completion of mitosis (40–42). Degradation of B type cyclins is essential for mitotic exit and cytokinesis (72, 73). Because DATS treatment caused accumulation of cyclin B1 in PC-3 cells (31), we postulated that DATS-treated cells might be unable to exit mitosis. The present study confirms that DATS-treated PC-3 and DU145 cells are arrested in M phase as evidenced by microscopic analysis of mitotic figures and increased Ser16 phosphorylation of histone H3. The ubiquitin-proteasome-mediated degradation of cyclin B1 is carried out by anaphase-promoting complex/Cdc20 and anaphase-promoting complex/Cdh1, the later acting in late mitosis (74). Reduced degradation of cyclin B1 may underlie the mitotic arrest observed in DATS-treated cells. However, further studies are needed to determine the mechanism of cyclin B1 accumulation in DATS-treated prostate cancer cells.

Another objective of the present study was to gain insights into the mechanism of DATS-induced Ser216 phosphorylation of Cdc25C (i.e. the kinase(s) responsible for phosphorylation of Cdc25C) and to determine its significance in cell cycle arrest caused by DATS. We focused on Chk1 and Chk2, which are intermediaries of DNA damage checkpoints (32–34, 51–53) for two main reasons. First, these kinases have been implicated in Ser216 phosphorylation of Cdc25C in response to different stimuli (33, 51). Second, Chk1 or Chk2 are known to be activated by pro-oxidants, such as ionizing radiation, and DATS causes reactive oxygen species generation in PC-3 and DU145 cells (31). The present study indicates that DATS-induced Ser216 phosphorylation of Cdc25C in prostate cancer cells is caused by Chk1 but not Chk2, although activating phosphorylations of both of these kinases are observed in DATS-treated prostate cancer cells (Figs. 2 and 8). This conclusion is based on our observations that DATS-mediated hyperphosphorylation of Cdc25C is inhibited by depletion of Chk1 protein but not Chk2 (Figs. 4 and 8). To our surprise, however, the Chk1 protein knockdown did not offer protection against DATS-induced G2-M arrest in either PC-3 or DU145 cells, although accumulation of Tyr15-phosphorylated (inactive) Cdk1 caused by treatment with DATS was significantly inhibited in Chk1-depleted PC-3 and DU145 cells. These results suggest that the Ser216 phosphorylation of Cdc25C in our model may not be sufficient to cause complete inactivation of Cdk1, perhaps because DATS does not affect Cdc25B level (31), and the cells, after a transient G2 block, are able to enter mitosis.

We were intrigued by our observations that Chk1, but not Chk2, protein knockdown offered partial yet statistically significant protection against DATS-induced M phase arrest in prostate cancer cells. During the preparation of this paper, the existence of a novel Brca-1 and Chk1-dependent mitotic exit checkpoint was reported in ionizing radiation-treated HeLa cells (75). These authors demonstrated that ionizing radiation-induced DNA damage not only delayed mitotic exit but also blocked mitosis in a Brca-1- and Chk1-dependent and securin-independent manner, leading to the appearance of cells exhibiting features of mitotic catastrophe (bi- and multinucleated cells) (75). Interestingly, we also observed multinucleated cells during microscopic examination of DATS-treated PC-3 cells (Fig. 1A). Thus, it seems reasonable to postulate that, similar to ionizing radiation, DATS treatment may activate a Chk1-dependent mitotic checkpoint, leading to mitotic catastrophe. Mitotic catastrophe, although not very well characterized, has been reported in cancer cells after treatment with anticancer agents (76, 77). Nonetheless, further studies are needed to determine the precise mechanism by which DATS treatment activates the mitotic checkpoint to cause mitotic catastrophe.

To summarize, our results indicate that DATS activates both G2 and M phase checkpoints in human prostate cancer cells irrespective of their p53 status. Furthermore, we provide experimental evidence to indicate that Chk1, but not Chk2, is involved in hyperphosphorylation of Cdc25C and Cdk1 in response to DATS treatment in PC-3 and DU145 cells. However, knockdown of Chk1 is not sufficient to abrogate G2 arrest in our model. On the other hand, Chk1, but not Chk2, seems to regulate DATS-induced mitotic arrest possibly through activation of the newly described mitotic exit checkpoint (75). We also observed features characteristic of mitotic catastrophe in DATS-treated PC-3 cultures. However, the relative contribution of mitotic catastrophe versus apoptosis, which we previously observed in DATS-treated PC-3 and DU145 cells (29, 30), to cancer cell growth inhibition by DATS is not clear and awaits further investigation. Nevertheless, to the best of our knowledge, the present study is the first published report to document mitotic catastrophe by a garlic-derived cancer chemopreventive agent.

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