Molecular and Cellular Biochemistry **288:** 107–113, 2006.
DOI: 10.1007/s11010-006-9126-6

Garlic compound, diallyl disulfide induces cell cycle arrest in prostate cancer cell line PC-3

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Received 26 October 2005; accepted 9 January 2006

Abstract

Prostate cancer is the most predominant cancer in men and related death rate increases every year. Till date, there is no effective therapy for androgen independent prostate cancer. Previous studies reported that aged garlic extract suppresses cancer growth. In the present study, diallyl disulfide [DADS], oil soluble organosulfur compound of garlic, was studied for its antiproliferative and induction of cell cycle arrest on prostate cancer cells *in vitro*. The suppression of cell growth was assessed by MTT assay. Induction of cell cycle arrest was assessed and confirmed by propidium iodide staining in flowcytometric analysis and western blotting analysis of major cell cycle regulator proteins. The results showed that DADS inhibited the growth of prostate cancer cells in a dose dependent manner, compared to the control. At 25 μM and 40 μM concentrations, DADS induced cell cycle arrest at G2/M transition in PC-3 cells. Western blotting analysis of cyclin A, B_1 and cyclin dependent kinase 1 [CDK1] revealed that DADS inhibited the cell cycle by downregulating CDK1 expression. It is concluded that DADS, inhibits proliferation of prostate cancer cells through cell cycle arrest. Dose dependent effect of DADS on PC-3 cell line was observed in the present study. (Mol Cell Biochem **288:** 107–113, 2006)

Key words: cell cycle arrest, cyclins, cyclin dependent kinase, diallyl disulfide, prostate cancer

Introduction

Prostate cancer (PCA) is the leading cause of death from cancer in older men and the most commonly diagnosed cancer in men overall [1]. For confined disease of low to moderately differentiated PCA, surgical or radiotherapy techniques are curative in the majority of individuals [2, 3]. Unfortunately, there is no effective cure for PCA once the cancer has spread beyond the pelvis; these patients are estimated to account for 30, 200 deaths in 2002 [1]. Although the rates of latent prostate cancer do not differ significantly throughout the world [4], there is wide variation worldwide in the distribution of clinical prostate cancer. Rates are much lower in the East (China and Japan) than in the United States [5]. Interestingly, when Japanese men migrate to the U.S. rates of clinical prostate cancer increase [6, 7], suggesting an environmental effect on prostate cancer incidence [8].

Epidemiological studies demonstrate an inverse correlation with prostate cancer incidence and increased consumption of garlic [9]. Epidemiological, clinical and laboratory studies have shown that crushed or processed garlic and their active principles, such as allicin, diallyl sulfide (DAS), diallyl disulfide (DADS) and diallyl trisulfide (DATS), give diverse biological activities,

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including antitumorigenesis, antiatherosclerosis, blood sugar modulation and antibiotics [10–13]. Garlic has been reported to reduce chemically induced esophageal, skin, pulmonary, stomach, colon, mammary and lung tumor [14–18]. Various mechanisms of action have been proposed to explain DADS anticarcinogenic effects. As far as the initiation phase is concerned, DADS has been shown to reduce the mutagenicity of N-nitrosopiperidine and benzo[a]pyrene as well as the hepatic DNA breaks induced by aflatoxin B1 or *N*-nitrosodimethylamine [19–21]. These effects could be related to the modulation of drug-metabolizing enzymes, which play a key role in xenobiotic activation as well as detoxication [22]. DADS enhances the activities, protein and mRNA levels of microsomal P450 1A2 and P450 2B1/2. It increases the activities of different phase II enzymes such as glutathione S-transferase, UDP-glucuronyl transferase and epoxide hydrolase [23–25]. Up to now, one explanation for DADS antipromoting effects has been its ability to retard the growth of established tumor cell lines, which has been demonstrated both *in vitro* and *in vivo* [26]. In particular, DADS has been found to be considerably more efficient than the water-soluble monosulfide S-allyl cystein in retarding the *in vitro* growth of human cells from colon, skin, breast and lung tumor cells [15, 27]. To explore other potential mechanisms of action, we focused our study on cell cycle inhibition and cell cycle regulator proteins on androgen independent prostate cancer cells.

Our previous studies showed that DADS could inhibit both androgen dependent and androgen independent human prostate cancer cell growth [28, 29]. In this study, we investigated the cell cycle progression and major regulators of cell cycle on the androgen independent prostate cancer cell line PC 3 after treatment with DADS.

Materials and methods

Chemicals

Diallyl disulfide [DADS], MTT [dimethyl thiazolyl tetrazolium bromide] and RPMI 1640 medium were purchased from Fluka Chemical Co. [USA], Propidium iodide was purchased from ICN chemicals, USA. Antiserum against cyclin B1, Cdc2 and β-actin antibodies were obtained from Merk, India. All other chemicals were from SRL chemicals, India.

Cell line and culture

Human prostate cancer cell line PC–3 was procured from National Center for Cell Science, Pune, India and cultured in RPMI 1640 medium with 10% fetal bovine serum [FBS], 1×10^5 cells were plated in each petriplates with RPMI 1640 medium containing 10% FBS. The cells were incubated for 12 h under 5% CO_2, 95% air at 37 °C.

MTT assay

MTT (dimethyl thiazolyl tetrazolium bromide) assay was performed by using the method of Yuan *et al*. (2004) [30] with slight modifications. Briefly, cells in suspension containing approximately 2×10^4 were added to each well of a 96-well culture plate and incubated for 24 h at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Diallyl disulfide was dissolved in dimethyl sulfoxide (DMSO) and mixed with culture medium and added to the cells in 24 well plates in five different concentrations (10 μM, 25 μM, 50 μM, 75 μM, 100 μM). Control cultures were treated with DMSO. The maximum concentration of DMSO added to the medium in this study was 0.01%. The 96-well culture plate was divided into 7 sections, with one section being treated by control culture media, the others were treated by one of the followings at 100 μL: culture media containing 10 μm, 25 μm, 50 μm, 75 μm and 100 μm of diallyl disulfide. The cultures were again incubated as above. After 24 h and 48 h, 100 μL of 1 mg/1 ml MTT solution was added to each well, and the cultures were further incubated for 4 h and then 100 μL of 20% SDS in 50% dimethyl formamide was added and the formed crystals were dissolved gently by pipetting 2–3 times slowly. A microplate reader was used to measure absorbance at 620 nm for each well. Growth inhibition rate was calculated as follows:

$$\text{Growthinhibition} = [A620/\text{nm of treated cells}/A620/\text{nm of control cells}] \times 100\%$$

Cell cycle analysis

Cell cycle analyses were performed by the method of Rasola and Geuna (2001) [31]. Briefly, cells were incubated in culture media alone or culture media containing reagents 25 μm and 40 μm of diallyl disulfide, at 37 °C for 24 h. Cells were harvested in cold PBS, fixed in 70% ethanol, and stored at 4 °C for subsequent cell cycle analysis. Fixed cells were washed with PBS once and incubated for 1 h in PBS containing 20 μg ribonuclease A per ml. Resuspended in 1 mL of PI staining reagent (50 μl propidium iodide in 1 ml of sodium citrate buffer, pH 7.4). 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 Beckon Dickinson FACS analysis system and quantitation of cell cycle distribution was performed using Multicycle Software (Phoenix Flow Systems, San Diego, CA). The percentage of cells in G1, S and G2/M phases were calculated.

Western blot analysis

Equal amounts of proteins from cell lysate were subjected to 10% polyacrylamide gels. Following electrophoresis, proteins separated on SDS–polyacrylamide gels were transferred to polyvinyldiene difluoride membranes [Millipore, USA]. To block the non-specific binding, membranes were incubated at 4 °C overnight with 5% skimmed milk powder, followed by a 12 h incubation at 4 °C with a antiserum containing antibodies against cyclin A, B1 or Cdc2. Peroxidase conjugated secondary antibody (1:10,000 dilution, from GENEI, Bangalore, INDIA), enhanced chemiluminense solutions from Perkin Elmer, USA was used to detect the immune reactive bands. The intensity of each band was determined using an Image analyzer (Quantity one software from Biorad).

Statistical analysis

Statistical analysis was performed using ANOVA and Student-Newman-Keuls test (Software Package for social studies SPSS) to determine significant differences among group means ($P < 0.05$).

Results

Cell viability assay

The effect of DADS on PC 3 cell viability was determined by MTT assay and the results are shown in Fig. 1. PC 3 cell viability was reduced significantly in both 24 h and 48 h treatment. In 100 μM concentration of DADS treatment, only 18.4% and 23.65% of cells were viable in 48 h and 24 h treatments, respectively. Percentage of cell survival was reduced gradually with increase in the DADS treatment concentration. From these results the IC_{50} value of DADS were calculated as $\approx$40 μM. So, for further studies we selected 40 μM IC_{50} to maximize the effects and a less concentration 25 μM as dose levels to identify the mechanism of action of DADS. These results indicated that the proliferation of PC 3 cells was significantly reduced with treatment of DADS, which is in a dose dependent manner.

Cell cycle analysis

The effect of DADS on cell cycle of PC 3 cells were analyzed by performing propidium iodide staining in flowcytometry. Consistent with its effect on cell growth inhibition, DADS induced cell cycle arrest significantly at G2/M phase in PC–3 cells (Fig. 2). 25 μM DADS treatment for 24 h resulted in accumulation of 30.62% of cells in G2/M phase of cell cycle. 40 μM DADS treatment for 24 h resulted in accumulation of 30.62% of cells in G2/M phase (Table 1). This suggests that there is a blockage in the G2/M phase transition, which may induce the cells to undergo apoptosis. Cells in the G1 and S phase were significantly reduced after 24 h treatment of 25 μM and 40 μM concentration of DADS. This shows that DADS in 25 μM and 40 μM concentrations reduces cell proliferation upto 43.38% and 33.8% respectively, and they were significantly lower than the control (72.94%) ($P < 0.05$) in G1 phase of cell cycle.

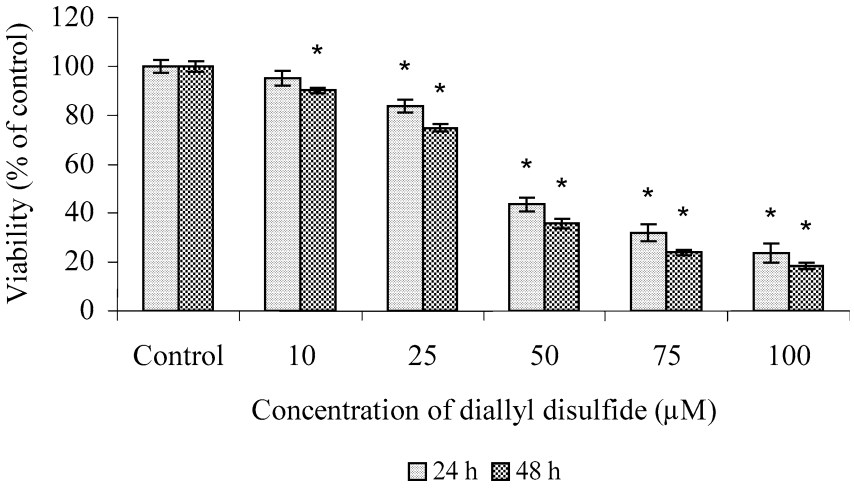

Fig. 1. Effect of diallyl disulfide on cell viability of PC-3 cells in 24 h and 48 h by MTT assay. Values represents % of control. Experiments were performed in triplicate and each value is mean ± SEM of three independent experiments. * represents significance between control Vs DADS treatment groups at $p < 0.05$ level using Student-Newman-Keuls test.

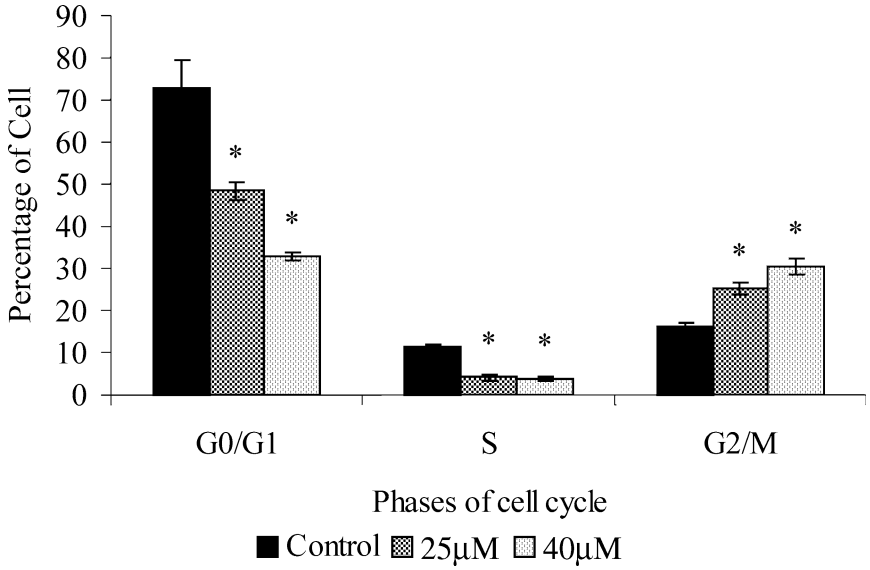

Fig. 2. Effect of diallyl disulfide on cell cycle of PC 3 cells assessed by flowcytometry after 24 h treatment. PC-3 cells were treated with diallyl disulfide at doses of 25 and 40 μM for 24 h in a 25 cm^2 flasks. After treatment, cells were trypsinized and suspended in 1 ml of fluorochrome solution [50 mg/ml propidium iodide, 1 mg/ml RNase A, 1.5% Triton X-100] for at least 1 h in the dark at 4 °C. Cell cycle analysis was performed using a Becton Dickinson flow cytometer, and quantitation of cell cycle distribution was performed using Multicycle software [Phoenix Flow Systems, San Diego, CA].

Protein expression of cyclins A, B1 and Cdc2

To further examine whether the protein expression of cyclins A, B_1 and Cdc2/CDK1 were modulated by DADS, an immunoblotting analysis were performed. Results showed that level of cyclin A in PC 3 cells was not affected by DADS treatment (Fig. 3a,b). As quantitated by densitometry, when PC 3 cells were treated with 25 μM and 40 μM concentration of DADS, the protein level of cyclin B1 was increased about 11.5% to 17.5%, respectively, as compared with the control. Cell cycle analysis revealed that the proportion of cells in the G2/M phase increased after exposure to 25 or 40 μM DADS. The ability of DADS, 25 and 40 μM concentration, to accumulate cells in G2/M phase of the cell cycle persists beyond its ability to increase cyclin B1 expression (Fig. 3a,b). A significant ($p < 0.05$) change in p34cdc2 was observed in 25 or 40 μM DADS treatment to PC 3 cells in culture, when compared to that of control. A trend towards a decrease (72.5% and 70.5%) in 25 and 40 μM concentration of DADS was observed, as a percentage of total p34cdc2 protein expression in cultures (Fig. 3a,b).

Table 1. Effect of DADS on cell cycle distribution of PC 3 cells assessed by flowcytometry after 24 h treatment. The PC 3 cells were incubated with 25 or 40 μM concentrations of DADS for 24 h, and the cells were harvested and analyzed for cell cycle and sub-G1 group and the percent of PC 3 cells in each phase are given in the table

Phase	Control	25 μM	40 μM
G0/G1	72.94 ± 6.8	48.38 ± 2.12*	32.80 ± 0.92*
S	11.23 ± 0.78	4.18 ± 0.62*	4.00 ± 0.52*
G2/M	16.05 ± 1.23	25.32 ± 1.54*	30.53 ± 1.95*

Values represented as percentage. Experiments were performed in triplicate and each value is mean $\pm$ SEM of six experiments. * represents significance between control Vs DADS treatment groups at $p < 0.05$ level using Student-Newman-Keuls test.

Discussion

DADS, an oil-soluble organosulfur compound and allyl mercaptan are formed in individuals eating raw garlic. The major component of cooked garlic is DADS, which is reduced to allyl mercaptan in blood [32]. DADS is reported to comprise about 60% of garlic oil [33], indicating that it is the most appropriate compound to use in the study of the possible effects of raw and cooked garlic. Previous studies showed that DADS suppressed the proliferation of androgen dependent and independent cancer cells such as PC-3 and LNCaP cells in culture [28, 29]. Additionally, Hong *et al.* [14] showed DADS-induced apoptosis in another two human lung cancer cell lines, H460 and H1299, with modulation of Bcl-2 proteins and p53 genes. In the present study, we found that DADS inhibits the growth of human PC 3 prostate cancer cells as shown by decrease in cell viability determined by MTT assay (Fig. 1). The growth inhibitory properties of DADS are likely attributed to its cell cycle arrest, at least partially, as indicated in Fig. 2 and Table 1.

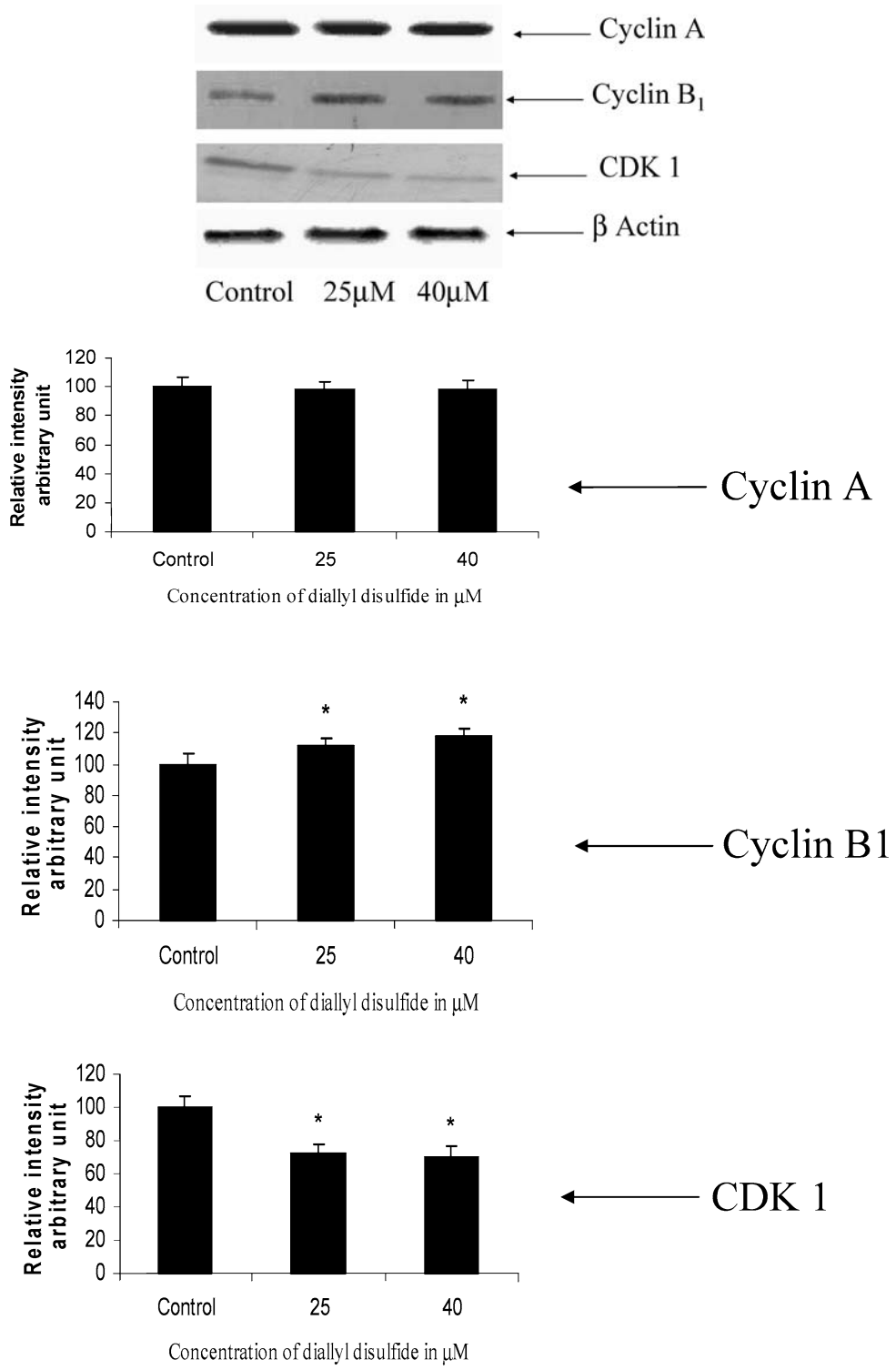

Fig. 3. (a) Protein expression pattern of Cyclin A, cyclin B1, p34cdc2 and β-actin after 24 h treatment with 25 or 40 μM DADS to PC 3 cells. Each value is mean $\pm$ SEM. All experiments were carried out three times and a representative blot is shown. (b) Quantitative data expressing the corresponding protein levels in PC 3 cells treated with control, 25 μM and 40 μM DADS group expressed in relative intensity arbitrary unit. * represents statistical significance between control Vs DADS treatment groups at $P < 0.05$ level using Student-Newman-Keuls test.

Suppression of proliferation of PC 3 cells by DADS is in a dose-dependent manner. In accordance with previous studies [34–36], a significant G2/M arrest of PC 3 cells could be observed when the cells were exposed to 25 μM and 40 μM DADS. This anti-proliferative effect indicates the possible value of DADS in therapy of prostate cancer. DADS treatment led to a significant cell cycle arrest at G2/M phase transition. Compared to the control, the percentage of cells at G2/M phase increased about 14.27% and 13.48% in 25 μM and 40 μM DADS treatment respectively as shown in Table 1. Previous studies showed that increased expression of p21 (waf1/cip1), activation of p38 MAP kinase and depressed p34cdc2 kinase activity may be involved in cell cycle arrest caused by DADS [34–37]. Consistent with other reports, DADS induced G2 to M transition arrest was clearly demonstrated in this present study also a reduction in the p34cdc2 level [36]. p34cdc2 is the key regulator of cell-cycle progression through G2-M [38]. In particular, activation of p34cdc2 kinase activity is required for progression from G2 to M. phosphorylation of the inhibitory residues Thr14/Tyr15 of p34cdc2 leads to decreased kinase activity and subsequent arrest at the G2/M phase [39]. The Cdc25C protein phosphatase is a key regulator of p34cdc2 phosphorylation status and kinase activity by dephosphorylating Thr14/Tyr15 residues [40]. *In vitro*, p38 binds and phosphorylates Cdc25C at serine 216 [41]. Phosphorylation of Cdc25C triggers cell-cycle arrest by the sequestration of Cdc25C by 14-3-3[42]. Yuan *et al.* [30] showed activation of p38 MAPK inactivated Cdc25C by phosphorylation of Ser216, thus initiating activation of the G2/M checkpoint, resulting in 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, expression of cell cycle regulator proteins, cyclin A, cyclin B_1 and p34cdc2, which regulates G2/M phase transition was reveled that DADS caused cell cycle arrest at G2/M phase by downregulating p34cdc2 levels with increase in cyclin B_1 expression.

Acknowledgements

The Financial assistance provided by University of Madras as UWPFEP Fellowship to one of the authors Mr. A. Arunkumar is gratefully acknowledged.

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