Growth Suppressing Effect of Garlic Compound Diallyl Disulfide on Prostate Cancer Cell Line (PC-3) in Vitro

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Prostate cancer is the most predominant cancer in men and prostate cancer related death increases every year. Till date, there is no effective therapy other than androgen ablation therapy. At this stage, induction of apoptosis is considered as a better strategy to control cancer. Previous studies reported that aged garlic extract suppresses cancer growth and enhances immune system against cancer. In the present study, diallyl disulfide, oil soluble organosulfur compound of garlic, was studied for its antiproliferative effect on prostate cancer cells in vitro. The suppression of cell growth was demonstrated by [3H]thymidine incorporation assay. Induction of DNA damage was assessed by agarose gel electrophoresis. The results showed that diallyl disulfide inhibited the growth of prostate cancer cells in a dose dependent manner, compared to the control. At 50 μM and 100 μM concentrations, diallyl disulfide induced DNA damage in PC-3 cells. It is concluded that diallyl disulfide, component of aged garlic extract, inhibits proliferation of prostate cancer cells through the induction of apoptosis.

Key words  diallyl disulfide; prostate cancer; apoptosis; DNA fragmentation; antiproliferation

Prostate cancer is the most invasive and frequently diagnosed malignancy, and second leading cause of cancer related deaths in the United States. Among them, approximately half of the patients develop incurable condition with no effective treatment. Current therapy for advanced prostate cancer is largely based on androgen deprivation. However, as with androgen ablation therapy, the responses are generally of short duration and resistant to therapy eventually results in progression. In this situation, control of prostate cancer through chemoprevention and intervention strategies is highly desirable. Induction of apoptosis in cancerous cells is considered to be very useful in the management and therapy of the disease. A wide variety of natural substances have been recognized to possess the ability to induce apoptosis in various cancerous cells.

Epidemiological studies suggest that garlic consumption is associated with a reduced risk of cancer incidence. These anticarcinogenic effects, as other biological properties of garlic are attributed to the presence of specific organosulfur compounds. Among these, diallyl disulfide is a major volatile organosulfur compound of garlic oil. Many studies on animal model showed its protective effect against chemically induced toxicity and also against carcinogenesis.

In the present study we investigated the effect of organosulfur compound of garlic, diallyl disulfide on the inhibition of proliferation and induction of apoptosis in human prostate cancer PC-3 cells.

MATERIALS AND METHODS

Materials  The PC-3 cell line was obtained from National Center for Cell Science, Pune. Diallyl disulfide, Ethidium bromide and Acridine orange were purchased from Fluka chemicals, U.S.A. Agarose was purchased from Pharmacia Biotech, U.K.

Methods. Cell Culture  PC-3 cells were cultured as monolayer in Minimal Essential Medium (MEM) containing 10% Fetal Calf Serum (FCS) in tissue culture flask. The media was supplemented with glutamine. It was maintained in humidified incubator in an atmosphere of 5% CO₂ in air at 37°C.

[3H]Thymidine Incorporation  Cell proliferation was assessed by thymidine uptake according to the method of Martin and Pattison. Diallyl disulfide was dissolved in dimethyl sulfoxide (DMSO) and mixed with culture medium and added to the cells in 24 well plates in four different concentrations (10, 25, 50, 100 μM). The dose was chosen from the previous studies. Control cultures were treated with DMSO. The maximum concentration of DMSO added to the medium in this study was 0.01%. After 24 or 48 h, the medium was removed and added 1 ml of medium containing 0.5 μCi of [3H]thymidine and incubated for 4 h. The radioactivity was counted in Amersham Pharmacia liquid scintillation counter.

Lactate Dehydrogenase (LDH) Assay  Lactate dehydrogenase activity was measured in both cell lysate and in the conditioned medium. After 24 h and 48 h incubation the cultured medium was taken separately and the attached cells were lysed by adding 0.1% triton X-100 and two cycles of freezing and thawing. The activity of LDH was measured by the method of King. Briefly, the substrate reaction buffer [0.5 mm lactic acid+0.1 n NaOH+0.1 m glycine buffer] was added to the cell lysate and medium. Dinitro phenyl hydrazine 0.02% was added as chromogenic agent and the absorb values (A) at 460 nm of reaction for 1 min were assessed. The percentage of LDH leakage was calculated as follows.

\[
\text{percentage of LDH leakage} = \frac{\text{activity in medium}}{\text{activity in cell lysate} + \text{activity in medium}} \times 100
\]

DNA Fragmentation  DNA fragmentation was done by using the method of Park et al., with slight modification. PC-3 cells were grown on petriplates and were treated with 10 μM, 25 μM, 50 μM and 100 μM concentrations of diallyl disulfide, for 24 or 48 h and trypsinized separately. 1×10⁶ cells were collected and centrifuged for 5 min at 250×g, and

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pellet lysed by the addition of 250 μl of lysis buffer. Proteinase K 10 μl was added and subsequently with, 10 μl RNase T1, left for overnight. 250 μl of phenol: chloroform was added and left for 2 h in the refrigerator, followed by 4 M NaCl, DNA was precipitated. The isolated DNA was separated on a 1.5% agarose gel and visualized by UV after ethidium–bromide staining.

**Ethidium Bromide and Acridine Orange (AO/EB) Staining** This staining was performed by the method of Spector et al., with slight modifications. 10 μl of cell suspension was added (0.5 × 10^6 to 2.0 × 10^6/ml) with 1 μl of AO/EB solution (1 part of 100 μg/ml of AO in phosphate buffered saline: 1 part of EB in phosphate buffered saline). Each sample was gently mixed. 10 μl of cell suspension was placed onto a microscopic slide, covered with a glass coverslip, and examined at least 300 cells in a fluorescence microscope at 20× objective.

**RESULTS**

**Inhibition of Cell Proliferation** PC-3 cells showed a significant decrease in [3H]thymidine uptake. Figure 1 shows the kinetics of proliferation from 24 h and 48 h diallyl disulfide treatment. At 100 μM concentration of diallyl disulfide treatment, the [3H]thymidine uptake was decreased to 76% and 81% in 24 and 48 h, respectively. A significant decrease in the proliferation for the concentrations 10 μM, 25 μM and 50 μM of diallyl disulfide were also observed to decrease at 32%, 60% and 66% in 24 h and at 39%, 68% and 77% in 48 h treatment (Fig. 1).

**Cytotoxicity Identified by LDH Leakage** The percentage of LDH release from cells was depicted in the Fig. 2. In all the 10 μM, 25 μM, 50 μM, and 100 μM concentrations, diallyl disulfide increased LDH leakage, which confirms the cytotoxic effect of diallyl disulfide.

**Induction of Apoptosis** To determine whether the inhibition of cell proliferation was due to apoptosis or necrosis, DNA fragmentation and morphologic changes in cell nuclei were examined. After 48 h of diallyl disulfide treatment, 50 μM and 100 μM doses caused DNA fragmentation in the PC-3 cells (Fig. 3). Thus, DNA from diallyl disulfide treated PC-3 cells at 50 μM and 100 μM concentrations displayed a characteristic damage pattern of DNA fragmentation on agarose gel electrophoresis. Figure 4 shows the acridine orange and ethidium bromide stained cells treated with diallyl disulfide. Diallyl disulfide treated groups showed increased number of apoptotic cells than that of control. Apoptotic cells increased with increasing diallyl disulfide concentration, at 10 μM concentration, apoptotic cells are 30.3 to 48.33% that is in 100 μM. Control has more live cells (Table 1). 10 μM, 25 μM, 50 μM and 100 μM of diallyl disulfide treatment showed high number of apoptotic cells with segmented nuclei. This indicates that diallyl disulfide induced apoptosis in prostate cancer cell lines in a dose dependent manner (as depicted in the picture).

**DISCUSSION**

According to previous studies, aged garlic extract inhibited the migration of rat sarcoma cells and suppressed the proliferation. They reported that this could be due to one or both of the active principles diallyl disulfide and allyl mercapto cysteine. Diallyl disulfide is an oil soluble organosulfur compound of garlic, which comprise about 60% of garlic oil, indicating that it is the most appropriate compound to use in the study. Sundaram et al., reported that organosulfur compounds in the processed garlic was able to suppress the growth of canine mammary tumor cells. Further, the antiproliferative activity of diallyl disulfide against colon (HCT-15), lung (A549), skin (SK MEL-2) and breast (MCF-7) cancer cells were also reported. In the present study, we confirmed the antiproliferative effect of diallyl disulfide on prostate cancer cell line PC-3 in vitro. In this in...
vestigation, diallyl disulfide reduced the cell growth up to 81% in 48 h treatment and 76% in 24 h treatment, which indicates the time dependent effect of diallyl disulfide.

Pinto et al.\textsuperscript{24} described the antiproliferative effect of other minor organosulfur compounds of garlic such as S-allylcysteine, S-allylmercaptocysteine and diallyl sulfide in LNCaP cells. Seki et al.\textsuperscript{25} studied the effect of garlic oil in HL-60 cells, where it significantly reduced the proliferation of HL-60 cells in culture. Takahashi et al.\textsuperscript{26} reported that diallyl disulfide inhibited the colon and renal carcinogenesis in rats. These reports showed strong antiproliferative activities of diallyl disulfide, so that we have taken the diallyl disulfide in our studies.

Diallyl disulfide is reported to decrease the $[^{3}\text{H}]$thymidine incorporation in human hepatoma cell line (Hep G2).\textsuperscript{27} This antiproliferative effect may be due to cell cycle arrest at progression of G2/M phase. Diallyl disulfide also inhibited the activity of p34cdc2 kinase activity in colon cancer cell line HCT-15.\textsuperscript{28} Knowles and Milner\textsuperscript{29} reported that diallyl disulfide, at 25 $\mu$M concentrations, up regulated the genes involved in cell cycle control.

LDH leakage is considered as a marker of cytotoxicity. LDH leakage assay monitors the integrity of the plasma membrane and is sensitive and easy to perform.\textsuperscript{30} In our present study, diallyl disulfide significantly increased the percentage of LDH leakage, which reveals the alteration in the plasma membrane integrity and permeability. Sheen et al.\textsuperscript{31} described that diallyl disulfide up to 1 $\text{mM}$ concentrations did not show any cytotoxicity to rat primary hepatocytes. The specific selectivity of cancer cells by diallyl disulfide has to be evaluated.

Apoptosis is an evolutionarily conserved suicide programme residing in all cells. It leads to cell death through a tightly regulated process resulting in the removal of damaged or unwanted cells. It also plays an important role in the development of various diseases including cancer.\textsuperscript{32,33} Recently interest has been focused on the manipulation of apoptotic process for the treatment and prevention of cancer. Much efforts have been directed towards the search for compounds that influence apoptosis.

Acridine orange is a vital dye and stains both live and dead cells. Ethidium bromide stains only cells that have lost membrane integrity.\textsuperscript{17} Live cells appeared uniformly green. Early apoptotic cells stained green and contain bright dots in the nuclei as a consequence of chromatin condensation and nuclear fragmentation. Late apoptotic cells also incorporated ethidium bromide and therefore stain orange but, in contrast to necrotic cells, the late apoptotic cells appeared condensed and often with fragmented nuclei. Hong et al.\textsuperscript{34} reported that in non small cell lung cancer cell line H460, diallyl disulfide at 5 $\mu$M concentration induced nuclear segmentation and apoptosis, but we observed, 10 $\mu$M as the effective dose concentration to induce nuclear segmentation. DNA fragmentation is a hallmark of apoptosis. In the present study, 25 $\mu$M and 100 $\mu$M concentrations, of diallyl disulfide induced DNA fragmentation, which confirms the apoptosis in PC-3 cells.

Diallyl disulfide induced apoptosis in breast cancer cells by increasing the expression of apoptotic protein Bax and reducing the expression of antiapoptotic protein Bcl2, and activating caspase-3, final caspase in the apoptotic pathway.\textsuperscript{15} The same mechanism could have been employed by diallyl disulfide in PC-3 cells also. However, it needs further confirmation studies.

In conclusion, we present evidence that diallyl disulfide, a major component of garlic, has growth inhibitory effect on prostate cancer cells and the effect was in dose and time dependent manner. The induction of apoptosis by diallyl disulfide may be the important mechanism by which its action

### Table 1. Effect of Diallyl Disulfide in Induction of Apoptosis on PC-3 Cells by Acridine Orange Ethidium Bromide Staining

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control</th>
<th>25 $\mu$M</th>
<th>50 $\mu$M</th>
<th>75 $\mu$M</th>
<th>100 $\mu$M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apoptotic cells</td>
<td>$18.0\pm1.6$</td>
<td>$91.0\pm4.5$</td>
<td>$116.0\pm3.6$</td>
<td>$120.0\pm7.4$</td>
<td>$136.0\pm8.9$</td>
</tr>
<tr>
<td>% of apoptosis</td>
<td>$6.0$</td>
<td>$30.3^*$</td>
<td>$38.6^*$</td>
<td>$40^*$</td>
<td>$45.33^*$</td>
</tr>
</tbody>
</table>

Each value represents mean±S.E.M. of five independent observations of 300 cells in 24 h diallyl disulfide treatment. * represents statistical significance of the data at $p<0.05$. Control vs. treated.
against cancer is based. However, it needs further investigation regarding the induction of apoptosis by the involvement of apoptotic and antiapoptotic proteins like Bax and Bcl2. Therefore, further studies are required to establish the molecular mechanism behind the antiproliferative effect of diallyl disulfide on prostate cancer cells.

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REFERENCES