Effects of DADS on cell proliferation and cytotoxicity
4.1 Introduction

Cancer is a challenging life threatening disease that causes extensive morbidity and wide mortality around the world. In the modern system of medicine, the advanced methods for treatment of cancers are surgery, intensive, endocrine, radiation therapy etc., which will develop the longevity in few cases, but at a substantial increase in health care costs patients morbidity and disability and will have relatively little effect on overall cancer incidence (Reizenstein et al., 1994).

The development of the most efficacious strategy for the prevention and treatment of cancers is based on understanding the underlying mechanisms of carcinogenesis. This includes the knowledge that the carcinogenic process is a multi-step, multi-mechanism process and that no two cancers are alike, in spite of some apparent universal characteristics, such as their inability to have growth control, to terminally differentiate, to apoptose abnormally and to have an apparent extended or immortalized life span. The multi-step process, involving the “initiation” of a single cell via some irreversible process, with the clonal expansion of this initiated cell by suppressing growth control and inhibiting apoptosis (promotion step), leads to a situation whereby additional genetic and epigenetic events can take place (progression step) to confer the necessary phenotypes of invasiveness, and metastasis (neoplastic stage). While it is clear that, in principle, prevention of each of these three steps is possible, in practical terms, while it would make sense to minimize the initiation step, one can never reduce this step to zero. On the other hand, since the promotion step is the rate limiting step of
carcinogenesis, intervening to block this step makes the most sense. Also, by understanding the ultimate biological function that confers growth control, terminal differentiation or apoptosis for cells, there is even some hope of treating some, but not all, malignant cells such that they can regain some ability to perform these vital cellular functions.

As there is a tremendous scope for the innovative methods of chemotherapy in the treatment of cancer, current systemic therapy is seldom effective, the effect is so marginal, incomplete responses and survival is shortened (WHO, 1994). The ultimate aim of cancer chemotherapy should be the use of compounds which selectively and specifically destroys the proliferating cancer cells, sensitively without noticeably affecting the growth, multiplication and survival of normal healthy cells.

A successful anticancer drug, which should kill or incapacitate the cancer cells without causing excessive damage to the normal cells. This criterion is difficult or perhaps impossible to attain. Hence, the cancer patients suffer unpleasant side effects while undergoing treatments. Therefore, the alternative medicine has evaluated to overcome this problem. Moreover, vast amount of synthetic work has provided relatively small improvements over the prototype drugs (Srivastava et al., 2003). Hence, there is a need for new prototypes and new templates for use in the design of potential chemotherapeutic agents. Natural products indeed are providing such template.
The efficacy of standard cancer therapy has reached a plateau for most solid tumors despite impressive progress in radiation therapy, such as dosimetry and more efficient methods of delivery of radiation doses to tumors, and in chemotherapy, development of novel drugs with diverse mechanisms of action on cell death and proliferation inhibition (Prasad, 2004).

The major obstacles of cancer chemotherapy are the development of drug resistance and the severe side effects. Due to the modest tumor specificity of many anticancer drugs, normal tissues are also damaged. This prevents the application of sufficient high doses to eradicate less sensitive tumor cell populations. Thereby, tumors develop drug resistance that leads to treatment failure. Novel strategies to broaden the narrow therapeutic range by separating the effective dose and toxic dose would be of great benefit for the improvement of cancer chemotherapy (Efferth and Volm, 2005).

Epidemiological studies suggests that garlic consumption is associated with a reduced risk of cancer incidence. These anticarcinogenic effects and other biological properties of garlic are attributed to the presence of specific organosulfur compounds. It has been extensively described in the review of literature. In the present study, the effect of DADS, one of the major organosulfur compounds of garlic on the growth suppression and cytotoxicity in human prostate cancer PC 3 cells were investigated.
4.2 Materials and Methods

4.2.1 Chemicals

Diallyl disulfide was purchased from Fluka Chemicals, USA. Acrylamide, bis-acrylamide, Ammonium persulfate, Bovine Serum Albumin (BSA), TEMED and RPMI (Roswell Park memorial Institute) – 1640 medium were purchased from Sigma Chemicals Co., USA. Sodium bicarbonate solution, trypsin EDTA, penicillin/streptomycin solution and amphotericin B were purchased from Biochrom, Germany. Fetal Bovine Serum (FBS) was purchased from Invitrogen Life Technologies, New Zealand. ^3^H- Thymidine was obtained from BRIT, Mumbai, India. MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) was purchased from ICN chemicals, USA.

p21\textsuperscript{cip1/waf1} (Rabbit polyclonal) was a kind gift from Dr. Martin Walsh, Mount Sinai School of Medicine, Newyork, USA and Dr. Luis Espinoza, Georgetown University School of Medicine, Washington DC, USA. cdc2/cdk-1 (Mouse monoclonal), cyclin A (Rabbit polyclonal) and cyclin B1 (Rabbit polyclonal) were purchased from Calbiochem, USA. Matrix metalloproteinases-2 (Rabbit polyclonal) and 9 (Rabbit polyclonal) were kindly provided by Prof. WG. Stetler-Stevenson, NIH, Maryland, USA. Histone H3 and H4 antibodies were a generous gift from Dr. Alain Verreault, Chromosome Dynamics Laboratory, London Research Institute, UK. PVDF membrane was purchased from Millipore, Bangalore, India. The secondary antibodies, Horseradish peroxidase (HRP)-Rabbit-anti mouse IgG and HRP-Goat-anti rabbit IgG were obtained from GENEI, Bangalore. Annexin-V
FITC binding assay kit was purchased from Sigma, USA. Caspase – 3 activity assay kit was purchased from Biovision, USA. Methanol and all other chemicals were purchased from SRL, India and were of analytical grade.

4.2.2 Cell culture reagents

1. **Roswell Park Memorial Institute (RPMI) 1640 Medium**

   10g of RPMI 1640 was dissolved in 800 ml of sterilized double distilled water. To this solution, 20ml of (1.5g/L) sodium bicarbonate was added. This was followed by the addition of 10ml of sodium pyruvate and 10ml of penicillin-streptomycin cocktail (100-units/ml penicillin and 100μg/ml streptomycin) and mixed thoroughly. The pH was adjusted to 7.4 using 1N HCl and 1N NaOH. It was then made up to 1000ml with double distilled water. Then the medium was sterile filtered using (0.22μ) membrane filter. The medium was then dispensed into sterilized container and stored at 4°C.

2. **Growth medium (RPMI 1640 with 10 % FBS)**

   100 ml of growth medium was prepared by adding 10 ml FBS in 90 ml MEM. It was stored in a sterile container.

3. **Phosphate Buffered Saline (PBS; pH 7.4)**

   0.63 g of sodium phosphate monobasic (NaH₂PO₄), 0.17 g of sodium phosphate dibasic (Na₂HPO₄) and 4.5 g of sodium chloride (NaCl) were
dissolved in 500 ml of sterile double distilled water. pH was adjusted to 7.4 with 0.1 N NaOH, sterile filtered (0.22 µ) and stored in refrigerator.

4.2.3 Cell culture

PC 3 cell line was procured from the National Centre for Cell Science (NCCS), Pune. The cells were grown in T-75 culture flasks containing RPMI 1640 supplemented with 10% FBS. Upon reaching confluence, the cells were detached using trypsin-EDTA solution.

4.2.4 Passaging the cells

The prostatic carcinoma cell line PC 3 was obtained from National Centre for Cell Science (NCCS), Pune. The cells were grown in culture flasks containing RPMI 1640 supplemented with 15% FBS. Upon reaching confluence, the cells were detached using trypsin-EDTA solution as follows:

The medium from the culture flask was aspirated. The flask was rinsed with 2 ml of PBS and aspirated again quickly. 1.5 - 2 ml of trypsin-EDTA solution was added and incubated at 37°C for about 3-5 minutes until cells started lifting. As soon as the cells were loose, using a transfer pipette the trypsinized medium containing cells was centrifuged at 1000 rpm for 5 min. the medium was carefully aspirated off. Care was taken not to put the pipette tip in the bottom of the tube, where the cells were pelleted. The cells were gently resuspended in fresh RPMI 1640 medium with 15% FBS by pipetting up and down 5-8 times. From the cell suspension, a drop was placed to the edge of the cover slip of Neubauer haemocytometer. The drop was let to run
under the cover slip by capillary action. Care was taken not to "force" the liquid and the entry of air bubble was avoided. Then the cells from the E₁, E₂, E₃, E₄ and E₅ squares were counted under microscope. The cells were then gently resuspended in fresh growth medium and transferred to sterile T-75 flasks and the volume of medium was made up to 10 ml with growth medium/flask.

4.2.5 MTT assay

Cell viability was assessed by MTT method described by Yuan et al. (2004) with slight modifications.

Reagents

1. MTT (0.5%): 0.25 g MTT was dissolved in 50 ml of serum free RPMI medium.

2. Solubilizing solution (20% Sodium Lauryl Sulfate (SDS) in 50% dimethylformamide DMF): 5.0 ml DMF was made up to 10 ml with Distilled water and 2 g of SDS were added and mixed well.

Experimental protocol for MTT assay

5 × 10³ cells were plated in 96 multiwell plates with RPMI medium containing 10% FBS. The cells were incubated for 12 h under 5% CO₂, 95% O₂ at 37°C. Then the RPMI 1640 medium with FBS was removed and replaced with SFM containing 1% BSA for 24h. After removing the BSA medium, the control plates received 0.01% DMSO containing medium and
treatment plates received 25, 50, 75, 100 μM of DADS containing RPMI-1640 medium. After 24 and 48 h the cells in the monolayer were used for cell proliferation assay. The treatment protocol is represented below:

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Wells</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>A₁ to H₁</td>
<td>Control</td>
</tr>
<tr>
<td>2.</td>
<td>A₂ to H₂</td>
<td>10 μM DADS</td>
</tr>
<tr>
<td>3.</td>
<td>A₃ to H₃</td>
<td>25 μM DADS</td>
</tr>
<tr>
<td>4.</td>
<td>A₄ to H₄</td>
<td>50 μM DADS</td>
</tr>
<tr>
<td>5.</td>
<td>A₅ to H₅</td>
<td>75 μM DADS</td>
</tr>
<tr>
<td>6.</td>
<td>A₆ to H₆</td>
<td>100 μM DADS</td>
</tr>
</tbody>
</table>

After 24 h, plates were harvested by staining with 100 μl of 0.5% MTT and then incubated at 37°C for 4 h. After incubation the stain was removed from the plate and 100 μl of solubilising solution was added, mixed well and after 2 h, the colour developed was read at 650 nm in an ELISA reader. The cell viability was calculated as percentage of viable cells and then plotted on a graph.

4.2.6 Cell proliferation

Cell proliferation was assessed by [³H] thymidine incorporation method (Martin and Pattison, 2000).
Reagents

1. PBS (pH 7.4)

2. 10% TCA

3. Lysis buffer (pH 8.2): 12.1 g of Tris and 7.4 g of EDTA were dissolved in one litre of double distilled water. The pH was adjusted to 8.0 and to this, 8 g of sodium dodecyl sulfate (SDS) was added.

4. Methanol AR

5. Thymidine [\(^{3}\text{H}\)]: 0.5 μCi/ml MEM

6. Scintillation fluid

   Scintillation fluid was prepared by mixing PPO (2,5-diphenyloxazole, 0.5%) and POPOP (2-p-phenylenes 5-phenyloxazole, 0.01%) in toluene

Experimental protocol for proliferation assay

5 × 10^4 cells were plated in 24 well multiwell plates with RPMI-1640 containing 10% FBS. The cells were incubated for 12 h under 5% CO\(_2\) and 95% air at 37°C. The control plates received 0.01% DMSO containing medium and treatment plates received 25, 50, 75, 100 μM of DADS containing RPMI-1640 medium. After 24 and 48 h the cells in the monolayer were used for cell proliferation assay. The treatment protocol is same as that MTT assay.
After drug treatment, medium was removed and 0.5 μCi of (³H) thymidine per ml was added and incubated for 2 h at 37°C. The cells were washed with ice cold PBS and incubated with 1 ml of 10% ice cold TCA for 30 min. The acid insoluble fraction was solublized in 0.5 ml of 0.1 N NaOH for 30 min at room temperature and the incorporation of (³H) thymidine into DNA was determined by scintillation counting. The results were expressed in % of cpm with reference to the control cells.

4.2.7 Assay of lactate dehydrogenase (EC 1.1.1.27)

The activity of Lactate dehydrogenase was assayed by the method of King (1965).

Principle

LDH catalyses the reversible reaction involving the oxidation of lactate to pyruvate with NAD serving as a cofactor. The reaction can be monitored with a colorimetric measurement. The determination of lactate dehydrogenase in the sample is based on the detection of NADH in the reaction. The change in the color is measured at 440 nm.

Reagents

1. 0.1 M Glycine buffer, pH 7.4

2. Buffered substrate: 4 g of lithium lactate was dissolved in 75 ml 0.1 N NaOH and was made upto 200 ml with glycine buffer.

3. NAD⁺ (5 mg/ml)
4. 0.02% Dinitro phenyl hydrazine (DNPH) in 1N HCl

5. 0.4 N NaOH

6. Stock Standard Tri sodium pyruvate: 11 mg of sodium pyruvate was dissolved in 100 ml of buffered substrate.

7. NADH solution (1 μM/L): 8.5 mg of NADH was dissolved in 10 ml of buffered substrate.

**Procedure**

1.0 ml of buffered substrate, 0.1 ml of medium, 0.2 ml of H₂O and 0.2 ml of NAD⁺ were added and incubated at 37°C for 15 min. Then 1 ml of DNPH was added and again incubated for another 15 min at 37°C. Then 0.5 ml of NaOH was added and the color developed was read at 440 nm within 60 seconds. For the standard curve, the test tubes labeled S₁ to S₅ is taken with a graded volume of Sodium pyruvate from 0.1 ml to 0.6 ml with the concentration ranging from 11 μg to 66 μg and all the above reagents were added except for the test sample NAD⁺ and were incubated for 15 min and then 0.5 ml of NaOH was added and the optical density was read at 440 nm. The activity of LDH was expressed as μmole of pyruvate liberated/min/mg of protein. The percentage of leakage was also found out.

**Calculation**

The activities of a Lactate dehydrogenase calculated with the following formula,
\[ \text{OD of unknown} \times \frac{1}{\text{Standard concentration} \times \text{Time correction}} \times \frac{\mu g \text{ protein}}{\text{factor}} = \mu \text{ mole of pyruvate liberated/min/mg of protein.} \]

The percentage of leakage was calculated as follows:

\[ \frac{\text{Activity in medium}}{\text{Activity in cell lysate} + \text{Activity in medium}} \times 100 \]

4.2.8 Statistical analysis

One-way Analysis of Variance (ANOVA)

The data were subjected to statistical analysis using one-way analysis of variance (ANOVA) adopted by Zar (1974).

In one-way ANOVA, the differences between means of more than 2 samples are tested for significance and are done by examining the variation within the whole groups of sample means. It consists of a comparison between two estimates of the overall variation (of the complete set of measurement included in the analysis): one estimate (treatment variance) being based on the variance of sample means about the grand mean and the other (error variance) based on the variance of the individual measurements about their treatment means. The ratio of these estimates is calculated as F and exceeds one, if sample means estimated differ from the population or group means.
The following prerequisites are necessary for calculating the analysis of variance:

(i) different treatment groups (T)
(ii) sample size of each group (n)
(iii) number of treatment groups (k) and
(iv) individual values (x)

The following steps were applied in the computation of the data.

1. Correction term, \( C = \frac{(\Sigma x)^2}{n} \)

   Represents squares of the sum of all values of x divided by the total number of values (n).

2. Total sum of squares, \( SS = (\Sigma x^2) - C \), representing subtraction of correction term (C) from the sum of squares of individual values of x (\( \Sigma x^2 \))

3. Sum of squares for treatments, \( SST = \frac{T^2}{k} \)

   Represents subtraction of the correction term from the sum of squares of a sum of each treatment (\( T^2 \)) divided by the number of treatment groups (k).
(4) Sum of squares for error, \( \text{SSE} = \text{SS-SST} \),

Representing subtraction of sum of squares for treatment (SST) from the total sum of squares (SS).

**Student-Newman-Keuls (SNK) test**

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>Degree of freedom (df)</th>
<th>Mean square Variance, ( \text{MS} = \text{SS}/\text{df} )</th>
<th>F test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between treatment groups</td>
<td>( (n-1) )</td>
<td>( \text{SST}/(n-1) )</td>
<td>( \text{MS between treatment groups} )</td>
</tr>
<tr>
<td>Within treatment groups (errors)</td>
<td>( k , (n-1) )</td>
<td>( \text{SSE}/k , (n-1) )</td>
<td>( \text{MS within treatment groups} )</td>
</tr>
</tbody>
</table>

The F value obtained was referred to F table against the df. When the F test was found to be significant, the data were subjected to Student-Newman-Keuls (SNK) test to assess the significance of individual variations between the treatment groups.

The multiple comparison procedure exemplified by the SNK test considers the null hypothesis \( \text{Ho: } \mu_B = \mu_A \), where the subscripts denote any possible pair of groups.

In SNK test, q value was calculated by dividing the difference between means...
Difference between means  
\[ q = \frac{\text{Mean Square Variance (MSV) within treatment groups}}{\text{Standard Error (S.E.)}} \]

S.E. was calculated using the following formula:

\[ \text{S.E.} = \sqrt{\frac{\text{Mean Square Variance (MSV) within treatment groups}}{n}} \]

where \( n \) is the number of observations in each treatment group.

If the calculated \( q \) value is equal to or greater than the critical value, from the table, then Ho: \( \mu_B = \mu_A \) is rejected.

In SNK test, the significance was considered at \( \alpha = 0.05 \) level.

**Standard error of mean (SEM)**

The SEM was calculated to analyze the variations among the observations in each treatment group and the data is expressed as mean ± SEM. The SEM was calculated by the following formula:

\[ \text{SEM} = \sqrt{\frac{\sum x^2 - [(\sum x)^2 / n]}{n (n-1)}} \]

where, \( x = \) individual observations, \( n = \) number of observations.
**Fig. 1** Effect of DADS on viability of PC 3 cells

Each value is mean ± SEM of six independent observations. * represents statistical significance between control and DADS treatment groups at $P < 0.05$ using Student Newman-Keuls test.
4.3 Results

Cell viability assay

The effect of DADS on PC 3 cell viability was determined by MTT assay and the results are shown in figure 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 was considered as ≈ 40 μM. So, for further studies 40 μM was given to maximize the effects and a less concentration 25 μM dose was used to identify the mechanism of action of DADS. The results indicated that the proliferation of PC 3 cells was significantly reduced with treatment of DADS and is in a dose dependent manner. In the remaining concentrations significant reduction was observed except 10 μM concentration of DADS treatment.

Inhibition of Cell Proliferation

PC 3 cells showed a significant decrease in [³H] thymidine uptake. Figure 2 shows the kinetics of proliferation from 24 h and to 48 h DADS treatment. At 100 μM concentration of DADS treatment, the [³H] thymidine uptake was decreased to 71.25% and 75.28% in 24 h and 48 h, respectively. A significant decrease in the proliferation for the concentrations 25 μM, 50 μM and 75 μM of DADS were also observed to decrease at 31.24%, 57.81% and
control and DADS treatment groups at \( p > 0.05 \) using Student Newman-Keuls test.

Each value is mean ± SEM of six independent observations. *Represents statistically significant difference between

48 hr

24 hr

Concentration of DADS in mM

<table>
<thead>
<tr>
<th>Concentration (mM)</th>
<th>100</th>
<th>75</th>
<th>50</th>
<th>25</th>
<th>10</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>HI thymidine uptake (percentage of cpm)</td>
<td>120</td>
<td>100</td>
<td>80</td>
<td>60</td>
<td>40</td>
<td>0</td>
</tr>
</tbody>
</table>

Fig. 2: Effect of DADS on proliferation of PC-3 cells.
Fig. 3  Effect of DADS on cytotoxicity of PC 3 cells

Each value is mean ± SEM of six independent observations. * represents statistical significance between control and DADS treatment groups at $P<0.05$ using Student Newman-Keuls test.
treatments. There was no significant change in the 10 μM concentration of DADS treatment in 24 h but in 48 h the proliferation was decreased to 10% (Fig. 2).

**Cytotoxicity identified by LDH Leakage**

The percentage of LDH release from cells was depicted in the figure 3. In all the 10 μM, 25 μM, 50 μM and 100 μM concentrations, DADS increased LDH leakage, which confirms the cytotoxic effect of DADS. The maximum leakage was observed in 100 μM treatment of DADS.
4.4 Discussion

DADS is an oil soluble organosulfur compound of garlic, which comprises about 60% of garlic oil indicating that it is the most appropriate compound to use in the study (Dausch and Nixon, 1990). Sundaram and Milner (1996b) reported that organosulfur compounds in the processed garlic were able to suppress the growth of canine mammary tumor cells. Further, the antiproliferative activity of DADS against colon (HCT-15), lung (A549), skin (SK MEL – 2), liver (HepG2) and breast (MCF-7) cancer cells were also reported (Sundaram and Milner, 1996b; Iciek et al., 2001). In the present study, the results confirmed the antiproliferative effect of DADS on prostate cancer cell line PC 3 in vitro.

Pinto et al. (1997) 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. (2000) studied the effect of garlic oil in HL-60 cells, where it significantly reduced the proliferation of HL-60 cells in culture.

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 (Molders et al., 1978). In our present study, DADS significantly increased the percentage of LDH leakage, which reveals the alteration in the plasma membrane integrity and permeability. Sheen et al. (1999) described
that DADS upto 1 mM concentrations did not show any cytotoxicity to rat primary hepatocytes. The specific selectivity of cancer cells by DADS has to be evaluated.

DADS has growth inhibitory and cytotoxic effect on prostate cancer cells and the effect was in dose and time dependent manner. Further studies were conducted to gain insights into the molecular mechanism on antiproliferation and cytotoxicity induced by DADS.