Chapter I

Cancer Chemopreventive and Apoptosis Inducing Potential of Diallyl Sulfide in Mouse Skin Carcinogenesis
Introduction

The use of alternatives to the medicines in the form of food and food products is an effective approach for the treatment of chronic diseases, including cancer. Thus, the intervention of chemopreventive strategies for controlling genetic diseases using dietary constituents provides a strong rationale to arrest or reverse the process of carcinogenesis before invasion and metastasis occur. During the past few years, cancer chemoprevention by dietary constituents has received a great deal of attention as a means of effective cancer control (Pezzuto, 1996; Martinez & Giovanucci, 1997; Kelloff et al, 1999). Studies on the tumor inhibitory compounds of plant origin have yielded an impressive array of novel structures. Besides, epidemiological studies suggest that consumption of diets containing fruits and vegetables may reduce the risk of developing cancer (El-Bayoumy et al, 1997; Reddy et al, 1997; Gescher et al, 1998). A number of dietary agents are also known to possess the anticancer properties in cancer cell lines and rodent bioassays at various sites including breast, prostate, colon and lung (Pezzuto, 1996; El-Bayoumy et al, 1997; Wargovich, 1997; Gescher et al, 1998; Greenwald et al, 2001). These include green and black tea polyphenols, I3C, sulforaphane, vitamin D, vitamin E, selenium and calcium (Kelloff et al, 1999).

In the recent past, a great deal of attention has been devoted to organosulfur compounds from garlic with potential chemopreventive properties (Fukushima et al, 1997; Pinto & Rivlin, 2001). One that has received considerable emphasis is DAS, a sulfur containing volatile compound in garlic (Allium sativum). Laboratory investigations provide sufficient evidence that it reduces the incidence of a multitude of chemically induced lung, skin, colon, esophageal and forestomach neoplasia (Wargovich et al, 1992; Hu et al, 1996a; Singh & Shukla, 1998 a & b; Yang et al, 2001). It has also been shown to inhibit aflatoxin B1 and NDMA induced liver preneoplastic foci in rats (Haber-Mignard et al, 1996). Several in vitro studies have also demonstrated its inhibitory effects on the tumor cells (Hageman et al, 1997; Hong et al, 2000). One of the classical models for evaluating the carcinogenic and anticarcinogenic potential of various test chemicals is the mouse skin model of carcinogenesis (Shukla et al, 1992; Slaga et al, 1995; Singh & Shukla, 1998 a & b; Marks & Furstenberger, 2000). The topical application of DAS has been reported to inhibit the development of tumors in both complete and two-stage model of mouse skin carcinogenesis by our laboratory (Singh & Shukla, 1998 a & b). DAS has also shown to possess antiproliferative effects.
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on the growth of transplantable Ehrlich ascetic tumor cells and inhibit angiogenesis in Swiss albino mice (Shukla et al, 2002). The possible mechanism for the antineoplastic activity of DAS has been suggested to be due to its antioxidant properties, capability of modulating several phase I and phase II enzymes and its antimutagenic potential (Guyonnet et al, 1999; Smith & Yang, 2000; Yang et al, 2001; Shukla et al, 2003). But thorough investigations into the molecular mechanisms of action of diallyl sulfide are still required. Several possible mechanisms are being explored by researchers to understand the mechanism of cancer preventive potential of DAS.

Apoptosis has been characterized as a fundamental cellular activity occurring under a wide variety of physiological and pathological conditions (Steller, 1995). The term “apoptosis” is a Greek word meaning “falling of leaves” and was originally defined to include certain morphological characteristics, including membrane blebbing, nuclear and cytoplasmic shrinkage and chromatin condensation. This event was first observed in a nematode larvae Caenorhabditis elegans, where this hermaphrodite worm undergoes this event during late embryogenesis in which normal cells apparently undergo apoptosis (Vaux et al, 1994). Apoptosis is also reported to play a key role in maintaining homeostasis in multicellular organism (Kerr et al, 1972). The outstanding feature of apoptosis is its remarkable stereotyped morphology showing condensation of nuclear heterochromatin, cell shrinkage and loss of positional organization of organelles in the cytoplasm (Kerr et al, 1972; Wyllie et al, 1980). It plays an essential role as a protective mechanism against neoplastic development by eliminating unwanted (genetically damaged cells or excess) cells that have been improperly induced to divide by a mitotic stimulus (Schwartzman & Cilowski, 1993; Thompson, 1995). It has been observed that the inherent capacity of the tumor cells to respond to apoptosis correlates with the expression of several oncogenes or tumor suppressor genes such as bcl-2, c-myc, ras or p53 and may be prognostic of the treatment (Compton, 1992; Wyllie, 1992; Schwaetzman & Cidlowski, 1993). The p53 gene, which is strongly implicated in animal and human carcinogenesis, is a significant regulator of the process of apoptosis (Stoner et al, 1997). While apoptotic pathway is related to induction of p53, this pathway is held in check by the anti-apoptotic gene bcl-2 (Reed, 1994). Apoptotic cell death can be distinguished from necrotic cell death (Table 8). In apoptosis damaged or abnormal cells appear to initiate their own program of death through the activation of endogenous proteases (Thornberry, 1997). This results to disruption in cytoskeleton structure, cell shrinkage and membrane blebbing.
Table 8: Morphological and Biochemical Characteristics of Apoptosis and Necrosis

<table>
<thead>
<tr>
<th></th>
<th>Morphological Differences</th>
<th>Similarities or confounding variables</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Apoptosis</td>
<td>Necrosis</td>
</tr>
<tr>
<td><strong>Nuclei</strong></td>
<td>Pyknosis and karyorrhexis (dense condensation of chromatin)</td>
<td>Karyolysis, preceded by irregular chromatin clumping</td>
</tr>
<tr>
<td><strong>Cytoplasmic organelles</strong></td>
<td>Intact</td>
<td>Disrupted</td>
</tr>
<tr>
<td><strong>Cell membrane</strong></td>
<td>Apoptotic bodies blebbing</td>
<td>Blebbing and loss of integrity</td>
</tr>
<tr>
<td><strong>Cell volume</strong></td>
<td>Cells shrink</td>
<td>Cells swell</td>
</tr>
<tr>
<td><strong>In tissues</strong></td>
<td>Single cells affected</td>
<td>Usually group of cells affected</td>
</tr>
<tr>
<td><strong>Tissue response</strong></td>
<td>No response</td>
<td>Inflammation</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Biochemical Differences</th>
<th>Similarities or confounding variables</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Nuclear DNA damage</strong></td>
<td>Nucleosomal and/or 50-300 kb fragments: ladders on agarose gels</td>
<td>Random: smears on gels</td>
</tr>
<tr>
<td><strong>Nuclear gene expression</strong></td>
<td>Usually needed</td>
<td>Not needed</td>
</tr>
<tr>
<td><strong>Mitochondrial DNA damage</strong></td>
<td>Spared</td>
<td>Occurs early</td>
</tr>
<tr>
<td><strong>Enzyme activation DNases Proteases Transglutaminase</strong></td>
<td>Necessary</td>
<td>Not necessary</td>
</tr>
<tr>
<td><strong>Membrane function</strong></td>
<td>Intact</td>
<td>Loss of function</td>
</tr>
<tr>
<td><strong>Cell internal milieu pH Ca²⁺ Na⁺/K⁺ pump</strong></td>
<td>Slightly acidic (pH 6.4) Often increases May be intact</td>
<td>Acidic Always increases Defective</td>
</tr>
</tbody>
</table>

(Source: Ramachandra & Studzinski, 1995)
Apoptosis also involves certain characteristic changes within the nucleus, which shows a characteristic laddering pattern of oligonucleosomal fragments. This results from inter-nucleosomal chromatin cleavage by endogenous endonucleases in multiples of 180 base pairs (Telford et al, 1991). This fragmentation is regarded as the hallmark of apoptosis. In cells undergoing apoptosis there is activation of a family of proteases called 'caspases', so named because they have an obligatory cysteine residue within the active site and cleave peptides adjacent to an aspartic acid residue (Thornberry, 1997). Activation of caspases appears to be directly responsible for many of the molecular and structural changes in apoptosis. The major biochemical and functional changes are shown in Fig. 6. Growing evidence from both in vitro and in vivo studies demonstrates that suppression of apoptosis is involved in tumor promotion by chemical agents (Taraphdar et al, 2001). Therefore, inhibition of apoptosis is one mechanism of tumor formation and many chemopreventive agents are known to act through the induction of apoptosis (Bursch et al, 1992; Lowe & Lin, 2000). Induction of apoptosis is, therefore being appreciated as an ideal way for the elimination of cancer cells and apoptosis inducing agents are being viewed as potential agents for the chemoprevention and for treatment of cancer (Taraphdar et al, 2001).

A number of naturally occurring compounds have been shown to possess inhibitory effects on the proliferation of tumor cells associated with induction of apoptosis (Katdare et al, 1998; Yang et al, 1998; Taraphdar et al, 2001). Certain phytochemicals are known to induce apoptosis in neoplastic cells but not in normal cells (Chiano et al, 1995; Hirano et al, 1995; Jiang et al, 1996a) (Table 9). It has become increasingly clear that apoptosis is an important mode of action for many antitumor agents including ionizing radiation (Radford et al, 1994), alkylating agents such as cisplatin and 1,3-bis (2-chloroethly)-1-nitrosourea (D'Amino & Mckenna, 1994), cytokine tumor necrosis factor (TNF) (Shih & Stuntman, 1996) and taxol (Gibb et al, 1997). Besides, I3C, curcumin, tea polyphenols and soy isoflavone genistin inhibits the aberrant proliferation by the regulation of cell cycle blockage and induction of apoptosis (Katdare et al, 1998; Yang et al, 1998; Khar et al, 1999). Gallic acid selectively induces cell death in various transformed cell lines such as PLC/PRF/5 (human hepatoma), HL-60 RG (human promyelocytic leukemia), P388 D1 (mouse lymphoid neoplasma), HeLa (human epithelial carcinoma) and dRLh-84 (rat hepatoma) (Inone et al, 1994). Curcumin, the major pigment in turmeric induces apoptosis in transformed rodent and human cells in culture (Jiang et al, 1996 a & b; Samaha et al, 1997). Resveratrol, a phytoalexin present in grapes and other food products, induces apoptosis in both solid and transplantable cancer cells by triggering
### Table 9: Phytochemicals Capable of Inducing Apoptosis

<table>
<thead>
<tr>
<th>Agent</th>
<th>Cell Line</th>
<th>Parameters</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genistein, Genistin, Daidzein, Biochanin A, Quercetin, Apigenin, Kaempferol</td>
<td>Promyelocytic Leukemia (HL-60), Prostrate Cancer (PCa, LNCGP, PC-3, DO-145), Lung Cancer (H-460), Bladder Cancer (HT-1376, UM-UC-3, RT-4, J-82, TCCSUP), Hepato-cellular Carcinoma (HCC), Colon Cancer (Caco-2, HT-29)</td>
<td>Cytotoxicity, Cell cycle arrest, Morphological characteristics</td>
<td>Gorezycg et al, 1993; Kuo, 1996; Davis et al, 1998; Wang et al, 1999; Zhou et al, 1999</td>
</tr>
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</table>

(Source: Taraphdar et al, 2001)
the CD95 signaling system (Clement et al., 1998), and decrease in bcl-2 expression (Surh et al., 1999). Zhou et al. (1998) reported that pure soy isoflavones such as genistein, daidzein and biochanin A, exhibited dose-dependent growth inhibition of murine and human bladder cancer cell lines through the induction of apoptosis. Oral administration of black tea is reported to inhibit proliferation and enhances apoptosis in both non-malignant and malignant epidermal tumors in two-stage mouse skin model of carcinogenesis (Lu et al., 1997). Black tea inhibited DNA synthesis and enhanced apoptosis of non-malignant as well as malignant tumor cells (Conney et al., 1999). Tea polyphenols, EGCG and ECG inhibits the growth of human lung cancer line PC-9 by inducing DNA fragmentation and reducing TNF-α level (Okabe et al., 1997; Suganuma et al., 1996, 1999).

Studies on the apoptosis inducing potential of allium derivatives have shown that allicin, an organosulfide from garlic possess inhibitory effects on the proliferation of tumor cells associated with induction of apoptosis (Zheng et al., 1997). Other organosulfides from garlic, such as DADS, DAT, suppress the in vitro proliferation of human A549 lung tumor cells through apoptosis (Sakamato et al., 1997). Hong et al. (2000) showed that DAS, DADS and garlic extract are effective in reducing the expression of anti-apoptotic genes (such as bcl-2) and the induction of pro-apoptotic genes (such as bax) and the tumor suppressor gene p53 in non-small cell lung cancer cell lines, suggesting that modulation of apoptosis associated cellular proteins may be the mechanism for apoptosis. However, not much information is available regarding the anti-proliferative activities of DAS via induction of apoptosis in solid tumors. Therefore, in present study, we investigated the antitumorigenic potential of DAS in mouse skin model of carcinogenesis and its mechanism of tumor suppression using apoptosis as a biomarker. The apoptosis inducing potential of DAS was investigated using multifacet approaches i.e. flow cytometry, terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) assay and formation of DNA ladder on agarose gels, a hallmark of apoptosis.

**Material and Methods**

**Materials:** 7,12-Dimethyl benzanthracene (DMBA), Propidium Iodide (PI), Ethidium Bromide and Diallyl Sulfide were purchased from Sigma (St Louis, USA). Apoptosis detection kit, Fluorescein was obtained from Promega. Medicons and Filcons were purchased from Beckton Dickinson, U.S.A. Rests of the chemicals used in the study were of analytical grade purity and were procured locally.
Animals: Swiss albino mice, male (*Mus musculus* L.) (body weight 10-12 gm) (Fig. 7a) were obtained from ITRC animal colony. Animals were kept for a quarantine period of one week under controlled temperature (22±2 °C) and humidity (55±5%) with 12 h light /dark cycle. Animals were fed a synthetic solid pellet diet (Ms Ashirwad Limited, Chandigarh, India Ltd.) containing all essential nutrients and water *ad libitum*.

Animal Bioassay: Mouse skin tumors were induced with DMBA as a complete carcinogen. For the induction of tumors, dorsal skin hair were clipped on the interscapular region over a 2cm² area (Fig. 7b) using electric clippers (Oster, USA) which were not lubricated with any oil or grease during the course of experiment. Only those animals which were found in the resting phase (telogen) of hair cycle initially were selected for the study. The animals were divided into five groups, comprising of 25 animals each. All the treatments were given topically on shaved dorsal skin as per dose and schedule given below:

Gr. I (Untreated) No Treatment
Gr.II (ACE+DMBA) 100μl Acetone applied topically followed 1 hr. later by 5 μg DMBA/animal in 100 μl acetone, 3 times/wk for 28 wks.
Gr.III (DAS+DMBA) 10 mg/kg b.wt. DAS applied topically in 100 μl acetone followed 1 hr. later by 5 μg DMBA/animal in 100 μl acetone, 3 times/wk for 28 wks.
Gr.IV. (DMBA+DAS) 5 μg DMBA/animal applied topically in 100 μl acetone followed 1 hr. later by 10 mg/kg b.wt. DAS in 100 μl acetone, 3 times/wk for 28 wks
Gr.V (ACE+DAS) 100μl Acetone applied topically followed 1 hr. later by 10 mg/kg b.wt. DAS in 100 μl acetone, 3 times/wk for 28 wks

Animals from all the groups were examined every week for gross morphological changes including body weight changes and development of tumors.
Figure 7: Swiss Albino Mice. a) Normal mouse; b) Normal mouse with shaved dorsal skin (Treatment site).
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locally on the skin during the entire study period. At the end of the study period i.e. 28 weeks all the surviving animals were sacrificed to check the development of tumors in the internal organs, if any. Skin from the painted area (with or without tumors) was excised and a part was fixed in 10% buffered formalin for 72 hrs. The formalin fixed tissue samples were dehydrated in ascending grades of ethanol (50%, 70%, 90% and 100%), cleared in xylene and embedded in paraffin. Tissue sections (5µ) were cut using microtome (American Opticals, U.S.A.), stained with haematoxylin and eosin and examined under the microscope (Leica, Germany). Histopathological classification of skin tumors was ascertained as described by Bogovski (1979). Part of the skin was snap frozen in liquid nitrogen and stored at −80°C in ultra deep freezer (Revco, USA) until required for flow cytometric analysis and genomic DNA isolation.

Flow Cytometric Analysis: The dorsal skin with or without tumors was washed in chilled phosphate buffered saline (PBS) and immediately transferred to ice. The fat layer of the skin was removed with the help of sterilized scalpel blade. The skin with or without tumors was chopped finely with the help of scissors and their single cell suspensions were prepared in sterilized Medicons (50 µm) by using Medimachine (Becton Dickinson, U.S.A) and were filtered through Filcons (30 µm). For flow cytometric studies, about 1x 10⁶ cells in approximately 1ml of suspension were pelleted (Ormerod et al, 1992), resuspended in 1ml PBS and fixed in 2ml 70% ice-cold ethanol. Cells were centrifuged (M.B Cooling centrifuge, India) at 5000 rpm for 10 min from the fixative and treated with Triton X-100 (0.2%) for 5 min. After incubation, cells were again centrifuged, resuspended in 1ml PBS, 100 µg/ml RNAse was added and incubated at 37°C for 30min. After further centrifugation, cells were resuspended in 1ml PBS and 50 µg/ml PI and incubated for 18 hrs. at 4°C. The data was acquired on Flow Cell Cytometer (BD-LSR, Becton Dickinson, USA) using Cell Quest and analyzed by Mod Fit LT 2.0 software.

DNA fragmentation assay: Genomic DNA was isolated from frozen skin/tumor tissue as per the method of Meniaitis et al (1989) with minor modifications (Ormerod et al, 1992). In brief, single cell suspensions of skin/tumor tissues were prepared individually and centrifuged (M.B. Cooling, India) at 10,000 g for 20 min at 4°C. The pellets so obtained were incubated for 30 min in lysis buffer (10 mM Tris-HCl, 10 mM EDTA and 4 M NaCl). RNA was digested with 2 µg/ml RNAse. Proteinase K was added in the medium to give the final concentration of 100 µg/ml and mixtures were incubated overnight at 37°C. After incubation, DNA was extracted with an equal
volume of equilibrated phenol. The two layers, aqueous and phenolic, were then mixed by inverting the tube gently to avoid shearing of the genomic DNA. After properly mixing the two layers for about 10 min, these layers were separated by centrifugation at 5000 rpm for 10 min at 4°C. The aqueous layer was then transferred carefully to a fresh tube and then equal volumes of Sevag Mixture (Phenol:Chloroform:Isoamyl Alcohol, 25:24:1(v/v)) was added and was again mixed gently and centrifuged. The aqueous layer was again transferred to a fresh tube and equal volumes of chloroform: isoamyl alcohol (24:1) was added, mixed and centrifuged. Aqueous layer was taken into a fresh tube and chilled ethanol (100%) was added in 2:1 ratio. The salt concentration was raised by adding 2M sodium acetate. The tubes were then kept for 1 hr at -20°C for the precipitation of genomic DNA. The precipitate was then spooled, washed with 70% ethanol, air dried and dissolved in appropriate amounts of TE (10mM Tris, pH 7.5, 1mM EDTA) buffer. The DNA was then quantitated spectrophotometrically (GENESYS™ 10 series, Rochester, USA).

Quantitation of DNA: The quantitation of DNA isolated from skin/tumor tissue was performed spectrophotometrically as per the procedure of Meniatis et al (1989) at 260nm and 280nm. The reading at 260 nm allows the calculation of the concentration of nucleic acid in the samples. An O.D. of 1 corresponds to approximately 50 μg/ml for double stranded DNA. The ratio between the readings at 260 and 280 nm (OD\textsubscript{260}/OD\textsubscript{280}) provides an estimate of the purity of the nucleic acid. Pure preparations of DNA have OD\textsubscript{260}/OD\textsubscript{280} values ranging between 1.8-2.0.

Agarose Gel Electrophoresis: The DNA (~2μg) was subjected to agarose (0.8%) gel electrophoresis (Bio Rad, USA) for 2-3 hrs. at 50 V in TBE buffer (45 mM Tris borate, 1 mM EDTA) consisting of 0.5 mg/ml ethidium bromide and visualized under UV light for DNA laddering using gel documentation system (Herolab, Gmbh, Germany).

TUNEL assay: Paraffin embedded sections of skin/tumor tissues were studied for their apoptotic status by utilizing the TUNEL assay, which detects fluorescein-12-dUTP labeled 3'-OH ends of genomic DNA. The apoptotic cells were detected in situ using Promega Detection System, Fluorescein as per manufacturer instruction (Fig. 8). Briefly, the tissue sections were deparaffinized in xylene twice for 5 min each and rehydrated in descending grades of ethanol (100%, 95%, 85%, 70% & 50%) for 3min each. Sections were then washed once with 0.85% NaCl, then with PBS and fixed with 4% methanol free formaldehyde solution in PBS for 15 min at room temperature. After
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Paraffin Embedded Sections

De-paraffinized in Xylene (twice)

Hydrate with descending grades of alcohol

Permeabilize with Proteinase K

Pre-equilibrate

Label DNA strand breaks with fluorescein - 12 - dUTP
(Add incubation buffer containing equilibration buffer, nucleotide mix & TDT enz.)

Termination of reaction
(2 X SSC)

Wash

Stain with Propidium Iodide

Analyze

(Propidium Iodide stains both apoptotic & non-apoptotic cells red. Fluorescein - 12 - dUTP gets incorporated at the 3'OH ends of the fragmented DNA, resulting in localized green fluorescence within the nucleus of apoptotic cells)

Figure 8: Schematic Representation of Terminal deoxynucleotidyl Transferase (TDT) mediated dUTP Nick End Labelling (TUNEL) Assay in Mouse Skin Tumors
fixing, sections were again rinsed twice with PBS each of 5 min duration. Tissue sections were permeabilized with 100 μl of the 20 μg/ml Proteinase K for 20 min, and again fixed in 4% methanol free formaldehyde solution in PBS with in between rinses of PBS of 5 min each. Then the sections were equilibrated with the equilibration buffer provided with the kit for 10 min at room temperature. After equilibration, 50 μl of TdT incubation buffer containing 5 μl nucleotide mixture and 1 μl TdT enzymes/section was added and the slides were then incubated at 37°C for 60 min inside a humid chamber in dark to allow the tailing reaction to occur. The reaction was terminated by immersing the slides in 2xStandard Saline Citrate (SSC) in a coplin jar for 15 min and the slides were washed thrice in PBS to remove the un-incorporated fluorescein-12-dUTP. Sections were stained with 1mg/ml propidium iodide solution for 15 min at room temperature in dark and washed in deionized water thrice. Apoptotic cells were visualized immediately under a fluorescence microscope (Leica, Germany) for the incorporation of fluorescein 12-dUTP at the 3'-OH ends of fragmented DNA, resulting in localized green fluorescence within the nucleus of the apoptotic cells. The apoptotic index is expressed as the amount of positive staining/ lesion. This was quantitatively assessed by estimation of the number and intensity of the stained cells/field using Leica image analysis system in 25 longitudinal sections.

**Statistical analysis:** Statistical analysis of the tumor data recorded was performed with student 't' test. P< 0.05 was considered significant. Differences in apoptotic index were compared using one-way ANOVA. F values with a probability < 0.05 were regarded as significant.

**Results**

**Animal bioassay:** During the entire course of study i.e. 28 weeks no increased rate of mortality or change in diet and water consumption was noticed in any of the group. The topical application of DAS resulted a significant protection in DMBA induced mouse skin carcinogenesis. The lesions at the site of application were observed. The lesions were confined to the painted area on the dorsal skin of mice and they were broadly classified as tumorous and non-tumorous lesions. The non-tumorous lesions were observed in the animals belonging to Gr. III & IV, which included initial loss of fur followed by poor hair growth at the site of application, hyperkeratinization and scaly skin. After about 8 weeks of exposure, a persistent baldness was observed in the painted area. The animals belonging to Gr. II (ACE+ DMBA) showed an almost similar
pattern of development of non-tumorous lesions as in Gr. III & IV but tumorous lesions also made their appearance in this group later on.

The tumors initially appeared as pinkish minute wart like growth developing into finger like projections firmly attached to the base with fragile tops. In due course of time, these tumors grow in size and were about 3-10 mm in height and 4-12 mm in diameter in few cases (Fig. 9a). There were also the signs of hemorrhages and necrosis in some of the tumors. Some closely related tumors were coalesced in due course of time. Some tumors showed inversion of the tissue at their places, characteristic of malignant conversion (Fig. 9b), mostly observed in positive control group (Gr. II, ACE+ DMBA). The tumor development was not observed in either Gr. V (ACE+ DAS) or Gr. I (untreated). This confirms that there was no spontaneous development of tumors or any toxicity associated with DAS exposure during the course of study.

The animal bioassay revealed a significant delay in the onset of skin tumorigenesis in DAS supplemented Swiss mice (Gr. III and Gr. IV) by more than three weeks in comparison to the animals exposed to DMBA alone (Gr. II) (Table 10). The induction of first tumor was observed on 53rd day of DMBA application in Gr. II but onset of tumorigenesis was recorded on 75th and 74th day in Gr. III and Gr. IV respectively (Table 10). The antitumor activity of DAS was also evident in the present set of investigations as 100% tumorigenesis was never achieved in DAS supplemented animals during the entire course of experiment (Table 10). Till the termination of the experiment, only 76% animals developed tumors in Gr. III and 80% animals showed growth on the back in Gr. IV (Fig. 10a). In DAS supplemented group 24 & 20 % of animals remained tumor free till the termination of experiment i.e. 28 weeks in Gr. III & IV respectively.

The multiplicity of tumors was also effectively inhibited by DAS application as the cumulative number of tumors was found to be significantly less in DAS supplemented animals in comparison to animals treated with DMBA alone (Fig. 10b). The average number of tumor per mouse was found to be about 3.0±0.5 and 4.5±0.6 in groups where DAS was given one-hour prior and after the DMBA treatment respectively, in comparison to 6.1±0.8 in DMBA exposed animals (Table 10). A comparatively higher incidence of mortality was recorded in Gr. II in comparison to Gr.

50
Table 10: Anticarcinogenic Effects of DAS in DMBA Induced Mouse Skin Tumors

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment</th>
<th>Number of Animals with tumors</th>
<th>1st Induction of tumor (in days)</th>
<th>Protection by DAS (%)</th>
<th>Total number of tumors</th>
<th>Avg. tumor/Mouse (Mean ± SE)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Non-malignant Tumors</td>
<td>Malignant tumors</td>
</tr>
<tr>
<td>I</td>
<td>Untreated</td>
<td>0/23</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>II</td>
<td>ACE + DMBA</td>
<td>16/16</td>
<td>53 (108)#</td>
<td>39 (40%)</td>
<td>58 (60%)</td>
<td>97</td>
</tr>
<tr>
<td>III</td>
<td>DAS + DMBA</td>
<td>16/21</td>
<td>75</td>
<td>27 (56%)</td>
<td>21 (44%)</td>
<td>48</td>
</tr>
<tr>
<td>IV</td>
<td>DMBA + DAS</td>
<td>16/20</td>
<td>74</td>
<td>43 (60%)</td>
<td>29 (40%)</td>
<td>72</td>
</tr>
<tr>
<td>V</td>
<td>ACE + DAS</td>
<td>0/22</td>
<td>-</td>
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* P<0.05 was considered significant over positive control (Gr. II).

# All the surviving animals developed skin tumors.
Figure 9: Mouse with skin tumors. a) Mouse with pedunculated squamous cell papillomas; b) Mouse with pedunculated squamous cell carcinoma
Figure 10: Tumor inhibitory effects of DAS in terms of reduction in a) Percentage of animals with tumors and b) Cumulative number of tumors.
Ill and Gr. IV, after about 23 weeks of exposure. The DAS application was also found to inhibit the malignant transformation of DMBA induced skin tumors (Table 10).

The histopathological examination of the tumors following hematoxylin and eosin staining revealed both non-malignant and malignant nature of tumors (Fig.11, 12). The majority of the tumors were benign skin papillomas, keratoacanthomas and certain tumors showing both papillomatous growth and acanthomous changes and hence was designated as ‘mixed type’ of tumors. Among the squamous cell papillomas about 70% of the tumors were pedunculated and remaining were flat type of squamous cell papillomas. Certain areas of the mouse skin became multilayered (6-8 layers) in place of normal 3-4 layers showing increased cell volume but the staining properties and other morphological features were remained unchanged. These areas are termed as ‘focal epidermal hyperplastic lesions’ (Fig.11b).

Pedunculated squamous cell papillomas were having a relatively thin stalk usually flexible at the base (Fig. 11c). In this type of tumors, the outgrowth from the skin surface consists of mainly three well defined layers: an inner layer of connective tissue or stroma, an intermediate layer of hyperplastic, relatively regular stratified squamous cell epithelium, and a peripheral layer of keratinized epithelial cells, that form a confluent keratin mass of variable, sometimes of considerable thickness. The stroma is mainly consists of connective tissue fibers and carries blood vessels thus regarded as ‘supply line’ of the tumors. The flat squamous cell papillomas showed almost the same pattern but here the breadth of the tumor was usually more than the height and had no stalk (Fig. 11d).

In keratoacanthomas the hair shaft of the hair follicles were replaced progressively by the horny masses of keratin. The striking feature of this type of tumor was the presence of keratin pearls within the tumors (Fig. 12a). In few cases, the tumors had both; an arboreal pattern of growth of squamous cells and horny masses of keratin developed from the hair element of the skin and hence were designated as mixed type of tumors (Fig. 12b).

The malignant epidermal tumors, squamous cell carcinoma (Fig. 12 c & d) showed very heterogenous structures. Polymorphism is commonly observed which is one of the markers of the manifestation of the malignancy. In few smaller tumors, increasing occurrence of atypism was also observed which is regarded as new population of the cells originating from a single nucleated cell. In squamous cell
**Figure 11:** Photomicrographs showing mouse skin/tumors (Hematoxylin and Eosin stained, magnification 128X)

a) Normal mouse skin
b) Mouse skin showing focal areas of hyperplastic transformations.
c) Flat squamous cell papillomas of mouse skin
d) Pedunculated squamous cell papillomas of mouse skin
Figure 12: Photomicrographs showing mouse skin tumors (Hematoxylin and Eosin stained, magnification 128X)

a) Keratoacanthoma showing large keratin pearls
b) Mixed type of tumor showing both papillomatous and keratoacanthomous growth
c) Squamous cell carcinoma
d) Squamous cell carcinoma at higher magnification (512 X)
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carcinoma usually confluent large, clusters and clumps of the cells were observed in few tumors. The irregularity of the cells was more evident in large tumors. Here, the nuclei of various size and shapes, from round to rod shape, having different staining properties were also observed along with many mitotic figures. In some cases, multinucleated cells, giant cells and cells under various stages of necrobiosis were also apparent.

**Flow Cytometric Analysis:** Flow cytometric analysis of mouse skin/tumors revealed the presence of a clear peak before G1 phase in tumor samples. The log was drawn against cell number (y-axis) and amount of the fluorescence detected on Argon 488nm laser. The untreated mouse skin cells showed a typical DNA histogram with majority of the cells in the G1 phase with persistent number of cells in the S and G2/M phase (Fig. 13a). In case of tumor cells of DMBA treated group (Gr. II), appearance of a small peak before the G1 peak was observed (Fig. 13b). The quantitation of the this peak was performed by using analysis software, ModFit 2.0 which revealed that 15.4% of the total cell populations were under the phase prior to G1 phase and hence were termed as 'sub G1' peak. This sub G1 peak is a characteristic of apoptotic DNA. The tumor cells obtained from Gr. III i.e. DAS+DMBA showed a distinct sub G1 peak indicating that 42.8% cells were showing the presence of apoptotic population (Fig. 13c). The application of DAS after DMBA administration (Gr. IV) showed no significant difference on its apoptosis inducing potential in comparison to Gr. III where DAS was given prior to DMBA. The quantitative analysis showed that the 34.6% of the total cells were under the apoptotic phase (Fig. 13d). Due to this increase in sub G1 cells, the percentages of G1, S and G2/M cells were significantly reduced in both the groups (Gr. III & Gr. IV)

**DNA fragmentation Analysis:** The DNA fragmentation analysis of mouse skin/tumors on agarose gels revealed a higher amount of apoptotic DNA in DAS supplemented groups (Gr. III & IV) (Fig. 14, lanes c & d). The nucleus of apoptotic cells has undergone internucleosomal chromatin cleavage by endogenous endonucleases in multiples of 180bp. This resulted in a characteristic laddering of oligonucleosomal fragments or ‘DNA ladder formation’, a hallmark feature of apoptosis on agarose gels. However, untreated mouse skin DNA did not show any oligonucleosomal fragmentation when subjected to agarose gel electrophoresis (Fig. 14, lane a). In tumors obtained from the Gr.II (ACE+DMBA), DNA fragmentation was also evident (Fig. 14, lane b). Although the extent of the DNA fragmentation was
Figure 13. Flow cytometric analysis of mouse skin tumor cells. Mouse skin tumor cells showed appearance of a sub G1 fraction i.e. characteristic of apoptotic cells which was more evident in DAS treated group. (a) Gr. I (Untreated), (b) Gr. II (ACE+ DMBA only), (c) Gr. III (DAS+DMBA), (d) Gr. IV (DMBA+DAS).
Figure 14: DNA agarose gel electrophoresis of mouse skin tumor cells. DNA was extracted from the skin/tumors as described in material & methods. About 2 µg of genomic DNA was loaded and electrophoreses at 40 volts. Induction of apoptosis (ladder formation) can be readily seen in DAS treated group. Lane M: Markers, a: Gr. I (Untreated), lane b: Gr. II (ACE+DMBA), lane c: Gr. IV (DMBA+DAS), lane d: Gr. III (DAS+DMBA),
significantly lowered in comparison to DAS administered groups (Fig. 14, lanes b, c & d). Therefore, it was confirmed that DAS could induce a significantly high incidence of apoptosis in mouse skin tumors.

**TUNEL Analysis:** Apoptotic status of skin tumors following DAS treatment by TUNEL assay showed induction of apoptosis in both non-malignant and malignant tumors of DAS treated groups (Fig. 15,16). The apoptotic index in nonmalignant tumors was $0.94 \pm 0.2$ in DMBA treated group (Gr II), which increased to $1.67 \pm 0.2$ and $1.58 \pm 0.2$ in DAS pre- (Gr. III) and post-treated (Gr. IV) groups (Table 11). Thus, DAS causes 78 and 68% increase in the TUNEL positive cells (apoptotic bodies) in nonmalignant tumors in comparison to the DMBA exposed group respectively (Fig. 15,16).

Examination of the nonmalignant tumors viz. focal epidermal hyperplasia, squamous cell papilloma and keratoacanthoma revealed 49%, 38% and 105% increase in the TUNEL positive cells, respectively in Gr. III and about 44%, 34% and 93% increase in Gr. IV. Similarly TUNEL assay showed about 94% and 82% increase in case of malignant tumors i.e. squamous cell carcinoma in Gr. III & Gr. IV respectively over Gr. II. The apoptotic indices for malignant tumors in Gr. III & Gr IV were found to be about $1.73\pm0.3$ and $1.62\pm0.27$ respectively compared to $0.89\pm0.2$ of Gr II (Table 11). However, no significant difference in apoptotic index was observed with TUNEL assay in both malignant and non-malignant tumors of the skin following DAS treatment (Gr. III & Gr. IV) (Table 11). The other morphological alterations like condensation of chromatin into crescent shaped caps, decrease in nuclear size, blebbing of the cytoplasm into small multiple apoptotic bodies were also evident in Gr. II, Gr. III & Gr. IV.

**Discussion**

The search for new chemopreventive and chemoprophylactic agents that are more effective and non-toxic has kindled great interest among researchers in organosulfur compounds from garlic (Fukushima et al, 1997; Pinto & Rivlin, 2001). Garlic is known for its therapeutic properties since ancient times and is probably the most widely studied medicinal plant (Agarwal, 1996). DAS, an active component of garlic responsible in part for its strong taste and odor is being viewed as a potential
Table 11. Effect of DAS on Apoptotic Index in Mouse Skin Tumors

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Apoptotic Index (Mean ± SE)</th>
<th>Non-Malignant Tumors</th>
<th>Squamous Cell Carcinoma</th>
<th>Total Tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Focal Epidermal</td>
<td>Squamous Cell Papilloma</td>
<td>Keratoacanthoma</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hyperplasia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACE + DMBA</td>
<td>1.58 ± 0.3</td>
<td>1.25 ± 0.4</td>
<td>0.82 ± 0.2</td>
<td>0.94 ± 0.2</td>
</tr>
<tr>
<td>DAS + DMBA</td>
<td>2.36 ± 0.4*</td>
<td>1.72 ± 0.4*</td>
<td>1.68 ± 0.2*</td>
<td>1.67 ± 0.2*</td>
</tr>
<tr>
<td>DMBA + DAS</td>
<td>2.27 ± 0.4*</td>
<td>1.67 ± 0.4*</td>
<td>1.58 ± 0.3*</td>
<td>1.58 ± 0.2*</td>
</tr>
</tbody>
</table>

* Values are significantly different (P<0.05) over positive control when analyzed by one-way ANOVA
Figure 6: Biochemical and functional changes during apoptosis
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Figure 15: Apoptosis inducing potential of DAS on DMBA induced mouse of skin tumors as evident by TUNEL assay. a) Effect of DAS on different types non-malignant skin papillomas, b) Effect of DAS on non-malignant vs malignant tumors.
Figure 16: Photomicrographs of skin/skin tumors showing increase in apoptotic bodies following DAS administration in TUNEL assay. (a) Gr. I (Untreated), (b) Gr. II (ACE+ DMBA only), (c) Gr. III (DAS+DMBA), (d) Gr. IV (DMBA+DAS).
anti-proliferative agent as has been shown to protect against a variety of chemically induced toxicity and carcinogenesis in animals (Wargovich et al., 1992; Haber-Mignard et al., 1996; Hu et al., 1996a). DAS is reported to inhibit the development of colon, esophageal carcinomas, pulmonary adenomas, skin papillomas/carcinomas and forestomach tumors in rodents when administered before carcinogen exposure (Wargovich, 1987; Sparnins et al., 1988; Wargovich et al., 1988; Wattenberg et al., 1989; Singh & Shukla, 1998 a & b). Epidemiological studies have shown that a lower risk of stomach cancer is associated with higher intake of *allium* vegetables in Shandong province of China (You et al., 1988; Han, 1993). Similarly an inverse relationship was observed between cooked garlic consumption and colon, rectal and esophageal cancers (Hu et al., 1991,1994; Gao et al., 1994; Giovannucci et al., 1994; Dorant et al., 1996). The mouse skin model of carcinogenesis has been widely used both for studies on mechanisms involved in chemical carcinogenesis and for testing of carcinogenic or anticarcinogenic agents of natural or synthetic origin (Berenblum, 1975; Shukla et al., 1988, 1992; Mukhtar & Agarwal, 1996; Singh & Shukla, 1998 a & b; Srivastava & Shukla, 1998). The results of the present study demonstrate the antitumorigenic potential of DAS in mouse skin carcinogenesis.

DAS has earlier been shown to possess antitumorigenic properties in complete as well as two-stage initiation promotion mouse skin carcinogenesis from our laboratory (Singh & Shukla, 1998 a & b). The results of the present investigation further lend support for cancer chemopreventive properties of DAS both by long-term carcinogenesis and mechanistic studies. An important feature of the study was that DAS suppressed the tumor in both the cases, i.e. given either one-hour prior to or after the carcinogen. However, somewhat greater degree of protection from chemically induced tumors was observed when DAS was given one hour prior to DMBA exposure (Table 10). Apart from its antitumor activity, DAS has been reported to ameliorate the toxicity of various xenobiotics including drugs viz cyclophosphamide (Goldberg & Josephy, 1987), ethanol (Morimoto et al., 1995) and acetaminophen (Hu et al., 1996a). The anticarcinogenic effects of DAS and other biologically active constituents of garlic was linked primarily to their antioxidant effects and induction of xenobiotic metabolizing enzyme systems (Guyonnet et al., 1999; Smith & Yang, 2000; Yang et al., 2001). Various natural compounds are known to exert their anticarcinogenic effects through their antioxidant activities (Ruby et al., 1995; Gerhauser et al., 1997; Frie & Higdon, 2003; Rietveld & Wiseman, 2003). Ruby et al (1995) investigated the antioxidant potential of natural curcuminoids, which indicates their potential use as chemopreventive agent in the inhibition of Ehrlich ascites tumor in mice. Protective
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effects of tea have also been well reviewed in various experimental and epidemiological studies (Frie & Higdon, 2003; Rietveld & Wiseman, 2003).

DAS is reported to cause selective inhibition of P450 2E1 activity, suppression of its level in microsomes and induction of P-450 2B1 in rats (Yang, 1993). Inhibition of azoxymethane induced colon carcinogenesis by DAS may be associated in part, with increased activities of phase II enzymes such as GST, NAD(P)H-dependent, QR, UGT in the liver and colon (Reddy et al., 1993). The anti-neoplastic activity of DAS, may at least in part be due to the elevation of all the three classes of GST (Maurya & Singh, 1991), GST-α and π forms mediate the conjugation of sulphydryl group of glutathione with toxic electrophiles and efficiently detoxify the electrophilic metabolites of carcinogen, which might be responsible for the suppression of neoplasia in mouse skin (Hayes & Pulford, 1995).

It is now well-established fact that majority of anticarcinogenic agents comprise a diverse group of compounds with different mechanisms of action, but their ultimate ability to induce apoptosis may represent a unifying concept for the mechanism of chemoprevention (Taraphdar et al., 2001). Therefore, the apoptosis inducing potential of DAS in solid tumors was worth to study in order to provide insight into its molecular mechanism of cancer chemoprevention. Consistent with the earlier reports on the mechanism of inhibition of allium derivatives via induction of apoptosis (Knowles & Milner, 1999), DAS in the present study showed a marked stimulatory effect on the apoptotic indexes in both malignant and non-malignant tumors as measured by TUNEL assay. TUNEL assay is being used successfully for the quantitation of apoptosis in paraffin sections especially for that tissue in which morphological determination is difficult such as tumor tissue (Rao et al., 1998; Yamamoto et al., 1998). This assay allows the detection of both double as well as single stranded DNA breaks by labeling the free 3'-OH termini with modified nucleotides (e.g. Biotin dUTP, DIG-dUTP, fluorescein-dUTP) in an enzymatic reaction.

The biochemical hallmark of apoptosis i.e. the extensive cleavage of the cell's DNA into oligonucleosomal sized fragments by a Ca^{2+}-dependent endonucleases, resulting in typical ladder patterns after DNA agarose gel electrophoresis (Arends et al., 1990), was also evident following DAS exposure in DMBA induced solid tumors (Fig. 14). The appearance of sub G1 fraction on flow cytometric analysis provides further support to our observation. In the literature, the presence of a sub G1 peak has only been found in association with apoptosis. It has been shown in cultures of murine
thymocytes (Nicoletti et al, 1991; Swat et al, 1991; Telford et al, 1991) and in a murine haemopoetic cell line (Rodriguez-Tarduchy et al, 1990) that the induction of apoptosis is accompanied by the appearance of a sub G1 peak in the DNA histogram. Earlier studies on other closely related organosulfides from garlic like DADS and DAT have been shown to suppress the in vitro proliferation of tumor cells through the induction of apoptosis (Sundaram & Milner, 1996; Sakamoto et al, 1997; Nakagawa et al, 2001). Ajoene and allicin, other active components of garlic have also been shown to possess inhibitory effects via induction of apoptosis in human promyeloleukemic cells, accompanied by generation of reactive oxygen species and activation of nuclear factor kappa (Zheng et al, 1997; Dirsch et al, 1998). S-allylmercapto cysteine, a stable thioallyl compound induces apoptosis in erythroleukemia, breast and prostrate cancer cell lines (Sigounas et al, 1997 a & b). Hong et al (2000) have shown that the effect of DAS, DADS and crude garlic extract on the expression of certain closely associated oncogenes bcl-2, bax and p53 in non small lung cancer cell lines and suggest that modulation of apoptosis associated cellular protein may be the possible mechanism for apoptosis.

Cellular apoptosis in concert with the proliferation and cyto-differentiation plays an important role in homeostasis (Steller, 1995; Jacobson et al, 1997). During carcinogenesis, aberrant proliferation disrupts growth control leading to down regulation of apoptosis (Cotter et al, 1990; Stewart, 1994). Genetic studies on apoptotic cell death have shown that positive regulation of p53 and negative regulation of bcl-2 play an integral role in mediating apoptosis (Miyashita et al, 1994; Silverstini et al, 1994). Thus, inverse correlation of the two gene products, together with upregulated cell cycle progression, may lead to aberrant hyper proliferation and carcinogenesis. It is noteworthy that an inverse correlation has been observed between p53 and bcl-2 expression in advanced breast cancers (Sierra et al, 1996), raising the possibility that the two genes may participate in a common pathway for the control of cell survival. The data generated from the present experiments showing induction of apoptosis by DAS in solid tumors therefore emphasizes the need for future experiments to focus on understanding the role of p53 and/or bcl-2 in the mechanisms for induction of apoptosis by DAS. The status of immunoreactivity to cell cycle regulatory and apoptosis specific gene products, together with analysis of gene expression at RNA and protein levels should elucidate the responsible mechanisms.

Thus from the present study we can conclude that naturally occurring phytochemicals present in garlic can significantly inhibit the development of chemically
induced solid tumors of mouse skin by inducing apoptosis. This is the first report showing mechanistic pathway of DAS mediated inhibition of chemically induced tumors by apoptosis using multifunctional approaches. The underlying mechanism(s) for inducing apoptosis of tumor cells by DAS needs more indepth research on the array of genes involved in regulation of apoptosis such as bax, bcl, p53 and p21. However, the results of the present study conclusively revealed that DAS could be used as a dietary adjuvant for controlling the neoplastic growth.