Chapter II

Chemopreventive effects of DADS on rat prostate intraepithelial neoplasia
9.1 Introduction

The prostate gland depends on androgens for growth, maintenance and function. In rodents, the prostate has clear lobulation. In rat, prostate can be divided into different lobes namely coagulating gland, dorsal, lateral and ventral lobes. The prostate gland and its secretions are required for the normal fertilization process. It is well known that prostate cancers are controllable by androgen deprivation therapy in early stages, but that androgen dependence is later lost, so lesions become incurable with hormone therapy. In experimental models, pharmacological doses of testosterone have been shown to increase development of naturally occurring or chemically-induced prostate carcinomas in rats. (Drago, 1984; Leav et al., 1988; Bosland et al., 1995). Shirai et al. (1991) reported that chronic administration of testosterone propionate (TP) at a pharmacological dose after 3,2'-dimethyl-4-aminobiphenyl (DMAB) induces invasive and partly metastatic adenocarcinomas, arising from the dorsolateral and anterior lobes, as well as the seminal vesicles. Previous work with immunohistochemical analysis of androgen receptors showed most ventral prostate carcinomas to be positive while more than 80% of invasive carcinomas arising from the dorsolateral prostate and seminal vesicle proved negative. (Shirai et al., 1995). Since normal glandular epithelial cells and atypical hyperplasias, regardless of the location in the accessory sex organs, are positive for androgen receptor (AR) immunohistochemistry, alteration in AR signalling might be a critical step for progression from atypical hyperplasias to invasive carcinomas. Orchidectomy after 20-week administration of testosterone to DMAB-treated animals
resulted in development of a few cases of invasive carcinomas by the end of the experiment, suggesting that androgen-independent lesions can indeed be induced (Shirai et al., 1994).

The rapid clinical development of new chemopreventive agents and dietary strategies for prostate cancer prevention is limited by the long periods of time and the great expense required to complete human prostate cancer studies. In addition, the slow progress in validating surrogate biomarkers that can be applied in short-term clinical studies has further inhibited the translation of laboratory and epidemiologic observations to clinical trials. Animal models of prostate carcinogenesis are rapidly being developed (Bosland, 1992; Xie et al., 2000; Martin et al., 2001) and provide tools for the pre-clinical assessment of the efficacy and toxicity of novel agents or dietary components. Rodent models also allow for the evaluation of biomarkers that can subsequently be used as surrogate endpoints in the initial short-term human studies that are necessary to define optimal dosing strategies for long-term definitive human prevention studies. The N-methyl-N-nitrosourea (MNU) androgen-induced model of prostate carcinogenesis in Wistar-Unilever rats has emerged as a valuable tool in this regard (Bosland et al., 1983; McCormick et al., 1998; McCormick and Rao, 1999). This model has several advantages including the development of a broad spectrum of histopathologic lesions corresponding to progression from hyperplasia to dysplasia and adenocarcinoma. Larger tumors are invasive, capable of metastasizing and emerge from the dorsolateral lobe and anterior prostate,
which are embryologically homologous to the site of origin of human prostate
cancer (Bosland and Prinsen, 1990).

It has been suggested that a change in the testosterone level with
advancing age is an important factor in initiation of benign prostatic
hyperplasia and prostate carcinogenesis (Ross et al., 1986). Despite extensive
research, the etiology of prostate cancer is not clearly defined. It is believed to
be multifactorial disease involving genetic, hormonal, dietary, age, race, and
environmental causes. The mechanisms leading to the initiation and
progression of prostate cancer are largely unknown. One of the reasons that
the progress has been slow is due to the lack of suitable animal models.
Although there are a number of animal carcinogenesis models, they are based
on single sex hormone, testosterone, or a combination of testosterone and
estrogen (Leav et al., 1988; Wong et al., 1998). The combination of
testosterone and carcinogen N-methyl-N-nitrosourea has the advantage of
inducing higher incidence of prostate carcinogenesis in Sprague-Dawly rats.
(McCormick and Rao, 1999; Liao et al., 2002). This particular model has
several advantages including the development of hyperplasia to dysplasia and
prostatic intra epithelial neoplasia in short term treatments (5 months).

Among various agents that have been tested for prevention of prostate
cancer, only a few have shown promising effects (McCormick and Rao 1999).
Due to the development of resistance against the available chemotherapeutic
agents, the evaluation of anticancer effects of various plant derived agents has
gained widespread attention in recent years. A positive outcome from such
studies could provide a scientific basis for their efficacy and usefulness in chemoprevention or chemotherapy of prostate cancer (Kelloff et al., 1999)

DADS has been demonstrated to exert a potential chemopreventive activity against human cancers including colon, lung and skin. DADS is an effective inhibitor of neoplastic CMT-13 canine mammary tumor cells in culture (Sundaram and Milner, 1996) and acts as an inhibitor of N-methyl nitrosourea induced mammary cancer (Schaffer et al., 1996). DADS inhibits proliferation of androgen independent prostate cancer cells through induction of apoptosis in vitro (Arunkumar et al., 2005). DADS was demonstrated to be effective in reducing the androgen dependent prostate cancer cells in culture (Gunadharini et al., 2005). However there is no report available on the role of DADS on prostate cancer initiation in in vivo model. Therefore the chemoprevention study was conducted to evaluate the effect of DADS in prostate carcinogenesis using Sprague-Dawly rats.
9.2 Materials and methods

9.2.1 Experimental design

Animals

Healthy adult male Sprague-Dawley rats weighing 150-180g (60 days old) were used in the present study. The animals were housed in clean polypropylene cages and maintained in an air-conditioned animal house with constant 12 hours light and 12 hours dark schedule. At all times during the studies, rats were permitted free access to food and drinking water. The animals were fed with standard rat pellet diet (Lipton India Ltd., Mumbai, India). Experiment was approved by the Institute animal ethical committee (IAEC-No 03/014/04)

Induction of prostatic intraepithelial neoplasia using carcinogen and hormone

Carcinogen and hormone exposure followed protocols published previously (Liao et al., 2002). First, each rats received daily i.p. injection of cyproterone acetate (50mg / kg b.wt.) (Sigma Chemicals, USA) for 21 consecutive days. One day after the last dose of cyproterone acetate, rats received daily i.p. injection of 100 mg testosterone propionate/kg body wt (Sigma Chemicals, USA) in 0.3 ml propylene glycol for 3 days. One day after the last testosterone propionate injection all rats received a single i.v. dose (50 mg/kg body wt.) of Methyl Nitroso Urea (MNU) (dissolved in saline at 10 mg/ml), through the tail vein. One week after MNU administration, rats
received daily *i.p.* injection of 2 mg/kg body wt. testosterone propionate/kg body wt for 60 days.

**Groups**

A total of 80 rats were divided into four groups. Each group consists of 20 animals.

**Group I**

Rats were received vehicle (propylene glycol) alone by intra peritoneal (i.p) injection.

**Group II**

Rats were received weekly twice the dose of 150 mg/kg body weight of DADS through oral gavage for 16 weeks.

**Group III**

Rats were induced prostate cancer by using carcinogen and hormone.

**Group IV**

Rats were induced prostate cancer and simultaneously treated with weekly twice the dose of 150 mg/kg body weight of DADS through oral gavage for 16 weeks.

Food intake and body weight change were monitored daily during the study. 20 weeks after the commencement of the experiment, all the animals
were sacrificed by cervical dislocation and fully necropsied. Ventral and dorsolateral prostates were removed from the adhering connective tissue and washed in physiological saline several times and weighed accurately. After thorough washing, ventral and dorsolateral prostates were fixed in 10% buffered formalin. The paraffin sections were stained with hematoxylin and eosin and examined histologically.

9.2.2 Histopathology

To investigate the histopathological changes in the prostate tissue, permanent mounts of the ventral prostate and dorsolateral prostate tissues were prepared as detailed by Jesik et al. (1982).

Principle

The formalin fixed tissues were washed and subjected to dehydration in ascending grades of alcohol. The tissues were blocked in paraffin wax, sectioned into ribbon and fixed on glass slides. After staining and destaining with hematoxylin and eosin, they were permanently mounted with DPX mount for histopathological studies.

Reagents

1. Phosphate buffered formalin (0.1 M; pH-7.4):

420 ml of 0.1 M disodium hydrogen phosphate was mixed with 0.1 M potassium dihydrogen orthophosphate until the pH was
adjusted to 7.4. A 10% (v/v) solution of formalin was prepared with this phosphate buffer.

2. Hematoxylin stain:

1 g of hematoxylin stain was dissolved in 10 ml of absolute alcohol and mixed with a solution containing 20 g of potassium alum previously dissolved in hot distilled water. After bringing this mixture to boiling point, 0.5 g of mercuric oxide was added. This mixture was cooled rapidly under tap water. This was then filtered and left to ripe for 10 to 15 days. This was used to stain the tissues.

3. Eosin stain (0.5%)

500 mg of eosin yellow powder was dissolved in 100 ml of distilled water and stored.

4. Absolute alcohol, AR grade

5. 30, 50, 70 and 90% alcohol

6. Xylene, AR grade

7. Paraffin wax (melting point 58-62°C), AR grade

8. Egg albumin
**Procedure**

Approximately, 5 to 10 mm\(^3\) of both ventral and dorsolateral prostate tissues, fixed in formal saline was washed thoroughly in running water. It was dehydrated in increasing order of alcohol (30, 50, 70, 90 and 100%) by placing the fixed tissues, for 30 min in these graded alcohols. The tissue was then transferred to xylene for clearing and subjected to cold infiltration at room temperature in a mixture containing paraffin wax dissolved in xylene for 30 min. The tissues were then hot infiltrated in oven at 58 to 60\(^\circ\)C in molten paraffin wax for 1 h. The hot infiltrated tissues were quickly removed and blocked quickly using metal blocks. The solidified blocks were trimmed to small size and sectioned using microtome to get ribbons of 8 to 10 micron thickness. The ribboned sections were placed on glass microscopic slides coated with egg albumin.

The microscopic slides were then exposed to decreasing order of alcohol (100, 90, 70, 50 and 30%) for 5 min each. The sections were dipped in hematoxylin stain for 5 min and then washed in running tap water for 3 to 5 min. After drying, the tissues were exposed to 0.5% eosin stain for 5 min and washed again in running tap water to remove the excess stain. The slides were dried and dehydrated in increasing order of alcohol (30,70, 90 for few seconds). After drying, the slides were cleared in xylene twice for 10 min. These cleared tissue sections were permanently mounted with DPX mountant. The permanently mounted sections of prostate were observed under light microscope for histopathological evaluations. The stained sections were
examined histologically for tumor types, using histological criteria described previously (Wong et al., 1998).

9.2.3 Statistical analysis

The data were analyzed using the SPSS 7.5 Windows Students version software. For all the measurements, one-way ANOVA followed by Student's Newman Keuls (SNK) test was used to assess the statistical significance of difference between control and DADS-treated groups. A statistically significant difference was considered at the level of $P<0.05$. 
Fig. 1 Effect of DADS on body weight of control and experimental animals

Each value is mean ± SEM of twenty animals. The T+MNU treated rats showed statistically significant decrease in body weight when compared with other groups. a: Control Vs T+MNU treated rats. b: T+MNU treated rats Vs DADS plus T+MNU treated rats at $P<0.05$ level.
Fig. 2 Effects of DADS on ventral and dorsolateral prostatic weights of control and experimental animals

Each value is mean ± SEM of twenty animals. The T+MNU treated rats showed statistically significant increase in ventral and dorsolateral prostate weight when compared with other groups. a: Control Vs T+MNU treated rats, b: T+MNU treated rats Vs DADS plus T+MNU treated rats at \( P < 0.05 \) level.
9.3 Results

Food intake, body weight and prostate weight changes

The mean food intakes of all the rats were equal. Control animals showed a gradual increment in their body weight throughout the experimental period. A strong suppression of body weight gain in T+MNU treated animals was observed. In the DADS treated group body weights were as that of control animals. Body weight starts decline from 10th week of the experiment and continued reducing constantly till the end of the experiment. But, DADS treatment reduced the body weight loss (figure 1) in the PIN (prostatic intraepithelial neoplasia) induced animals. Here the body weight represented at five week intervals rather than every week.

The ventral and dorsolateral prostatic weights were also increased in the T+ MNU treated group. DADS treatment reduced the organ weight in these lobes significantly from that of T+ MNU treated group rats. DADS alone treated group did not show any change (figure 2).

Histopathology

Survival was limited to 20 weeks from the start of the treatments. PIN regions were found in 8 (40%) animals out of 20 in the dorsolateral prostate lobe and 7 (35%) out of 20 rats were developed PIN in the ventral lobe of the prostate. The 4 -7 PIN regions were found in each of the T+ MNU treated rats. About 10% (2 out of 20) of animals developed PIN in the ventral and dorsolateral lobes of DADS treated group. Dysplasia was observed in 11 (55%) rats in the dorsolateral lobe. More dysplastic loci were found in
Fig. 3 A. Histopathological appearance of normal dorsolateral prostate. A single layer of cuboidal cells lines the tubules with secretion in the lumen. Epithelial tubules, surrounded by a thin layer of smooth muscle cells, are distributed within the loosely organized stroma. Magnification x 200, H & E.

Fig. 3 B. T + MNU treated rats. Prostatic intraepithelial neoplasia in DLP. Multiple dysplastic and hyperplastic sites were seen within the same glandular epithelium. It shows the papillary formations within the lumen. Magnification x 200, H & E.

Fig. 3 C. DADS plus T + MNU treated rats. Hyperplasia and dysplasia are less common and loosely organized stroma was seen. Absence of dysplastic and hyperplastic nodules with normal stromal compartment is also observed. It also shows cell death of epithelial cells. Magnification x 200, H & E.

E – Epithelium, L – Lumen, S – Stroma
Fig. 3 Effect of DADS on rat dorsolateral prostate histology of control and experimental animals

Dorsolateral prostate

(A) Control

(B) T + MNU treated

(C) DADS plus T + MNU treated
Fig. 4 A. Normal rat ventral prostate (VP). The tubules are lined by a single layer of cuboidal cells composed of columnar cells. Magnification x 200, H & E.

Fig. 4 B. Multiple dysplastic and hyperplastic sites were seen within the same glandular epithelium. Fully developed prostatic intraepithelial neoplasia, precursor of prostatic carcinoma in VP with loss of basal epithelial cells. Magnification x 200, H & E.

Fig. 4 C. Ventral prostate of DADS plus T+ MNU treated rats. Note that absence of dysplastic and hyperplastic nodules, stroma is very loosely organized. Magnification x 200, H & E.

E – Epithelium, L – Lumen, S – Stroma
Effect of DADS on rat ventral prostate histology of control and experimental animals

Ventral prostate

(A) Control

(B) T + MNU treated

(C) DADS plus T + MNU treated
T+MNU treated group. In the ventral lobe 60% (12) rats were developed hyperplasia and 50% (10) rats were developed dysplasia in the T + MNU treatment group. In the dorsolateral region, 13 (65%) rats were developed hyperplastic changes. In the DADS treated group, occurrence of hyperplasia and dysplasia were less in ventral (15% hyperplasia and 10% dysplasia) and dorsolateral (20% hyperplasia and 10% dysplasia) lobes. The hyperplastic, dysplastic and PIN region, frequency of occurrence were reduced in the ventral and dorsolateral lobes of DADS treated group significantly, from that of control, normal diet fed animals (figure 3 and 4).
9.4 Discussion

The results of the present study showed that DADS, an organosulfur compound of garlic has significant potency as an inhibitor of cancer induction in prostate gland of Sprague Dawly rats. Epidemiological studies showed that enhanced garlic consumption is closely related with reduced cancer incidence including prostate cancer (Sparnins et al., 1988; Buiatti et al., 1989; Dorant et al., 1993; Key et al., 1997). There are several reports demonstrating the anticancer activity of DADS in several chemically induced cancers of different organs like colon, lung, mammary and skin (Wargovich, 1987; Haber-Mignard et al., 1996; Munday and Munday, 1999). Schaffer et al. (1996) reported that DADS prevented the MNU induced mammary cancer in animals. Munday and Munday (1999) showed that at this 150 mg / kg b.wt. dosage level, there was a significant increase in the phase II enzymes in rat gastric tract. At this dose level DADS exerts protection against gastrointestinal tract cancer. Guyonnet et al. (2000) stated that at this dosage level DADS induces significant increase in the isoenzymes of phase I drug metabolizing enzymes. The protection against cancer may arise from several mechanisms including inhibition of the formation of chemical carcinogen, decrease of activation and/or increase of detoxification of carcinogens, induction of cell cycle arrest and apoptosis (Sundaram and Milner, 1996b; Le Bon and Siess, 2000; Knowles and Milner, 2000b). General findings suggest that the MNU model is relevant to human disease (Liao et al., 2002). PIN is a lesion that is now widely accepted as a pre-malignant condition for prostate cancer development (Brawer, 1992; Lipski et al., 1996). DADS had
previously demonstrated to possess anticarcinogenic activity in other tissues like breast, colon and gastro intestinal tract (Wargovich, 1987; Haber-Mignard et al., 1996; Munday and Munday, 1999). In T+MNU treated animals, more number of PIN and adenoma loci were observed, which shows the induction of prostate Tumor, PIN is primarily accepted as a morphologically identifiable early stages in prostate cancer (Foster et al., 2000). Two types of PIN were identified in the animals. Multi layer of dysplastic cells which rise on the epithelial layer and dysplastic cells forms papillary structures. DADS treated cells showed PIN morphology more or less seen in T+MNU treated rats but their occurrence was much less than that of hormone plus MNU treated animals. Tumors occur preferentially at DLP than VP. Although Christov et al. (2002) indicated that PIN could be used for assessing the efficacy of chemopreventive agents on prostate carcinogenesis; additional studies are needed to assess the biology of PIN and its progression toward malignant tumors. In the present study, DADS treatment showed significant reduction in PIN, hyperplastic and dysplastic loci.

In conclusion, the present investigation provides evidences for the chemopreventive effect of DADS on prostate carcinogenesis. Further studies are required to identify the molecular mechanism of DADS involved in the chemoprevention of prostate carcinogenesis.