CHAPTER 3

In search of a novel agent that drives the prostate cancer cell lines to undergo apoptotic cell death.
**Introduction**

SRD5A2 plays an important role in male developmental biology, physiology, and pharmacology. The Steroid 5α-reductase enzyme was initially characterized in the 1950s in rat liver slices based on its ability to convert deoxycorticosterone to five α-reduced metabolites. Steroid 5 alpha-reductase is a NADPH dependent enzyme responsible for the reduction of testosterone (T) into the more potent androgen dihydrotestosterone (DHT) [75]. This enzyme is located in androgen-dependent tissue such as prostate, seminal vesicles, epididymis and other reproductive tissues [75]. It has been determined that DHT interacts more efficiently with the androgen receptors than its precursor T [111] and has been implicated in the pathogenesis of prostate cancer, benign prostatic hyperplasia (BPH), acne and male pattern baldness [111, 112]. This fact indicates that both the 5 alpha reductase enzyme and DHT play important physiological and pathological roles in human males. Therefore, the inhibition of DHT formation by inhibition of 5-alpha reductase enzyme is a treatment for androgen-dependent afflictions [113]. The most extensively studied class of 5 alpha reductase inhibitors are the 4-azasteroids [114], which include the drug finasteride. First, developmental studies showed that the activity of 5α-reductase in mammalian embryo was highest in the primordia of the prostate and external genitalia prior to their Virilization, but very low in wolffian duct structures. [100]. This compound is the first 5α-reductase inhibitor approved in the USA for the treatment of BPH. In humans, finasteride decreases prostatic DHT levels by 70–90% and reduces prostate size, while testosterone tissue levels remain constant [115]. It has been claimed that 5αR inhibitors decrease prostate volume and induce atrophy in benign prostatic epithelium and cancer [65]. Finasteride inhibits the LNCaP cell growth in a dose dependent manner [51]. FZ was one of those few compounds elicited a profound growth inhibitory activity on prostate cancer cell growth lines both in vitro and in vivo. The possible mechanism of a lead compound has been examined in silico using various tools and techniques available as discussed in previous chapter.
RESULTS AND DISCUSSION

Growth inhibitory effect of selected compounds in cell culture

In an attempt to identify new anticancer agents that are cytotoxic to cancer cells, but not to normal cells, a panel of small molecule drugs has been screened (Chembridge, Inc., San Diego, CA). Few lead compounds were identified and awaited for its characterization earlier in our laboratory. Meanwhile, another thirty five small molecule analogues of finasteride, were made available to us by Prof. L M Bharadhwaj from the Pharmacy department, Panjab University. In order to get a more potent drug related to Finasteride, these in house synthetic analogues were initially screened for their cytotoxicity using mouse peritoneal macrophage cells. Freshly isolated macrophage cells ($10^5$) were seeded in triplicate in 96 well plates in DMEM supplemented with 10% FCS with standard antibiotics and cultured for 48h at 37°C as mentioned in the Materials and Methods section. Cells were allowed to attach for three hours and then, unattached dead cells and contaminating lymphocytes were washed off and the cultures were fed with fresh complete medium. Next day cells were treated with drugs with varied concentrations. Drugs were solubilized in DMSO (cell culture grade). The concentration of DMSO was adjusted so that it did not exceed 1% in any case. Treated cells, along with untreated and DMSO mock controls were cultured for 48h at 37°C with 5% CO$_2$. Standard mitochondrial enzyme assay (MTT) was performed to assess the viability of the cells. Data obtained from colorimetric measurements was plotted as shown in Fig 18a. Out of thirty two compounds tested majority of them were toxic to the macrophage cells. Some of these compounds with least toxicity were further tested for their growth inhibitory effect on human prostate cancer cell lines (DU145 and PC3), but no significant growth inhibition was observed with any of these compounds (data not shown) except one.
Figure 18: Cellular toxicity of thirty finaestride analogues were examined. Toxicity studies were done using normal mouse peritoneal macrophages cells in culture. After a 48h treatment with various small molecules concentration ranging from 0.01 to five μg/ml cell viability was assessed by MTT assay. Data represent the means ± S.D. from at least three measurements.

Fortunately, we have identified a compound called Methyl 5-[phenylthio]-2 benzimidazole carbamate (FZ) which showed marked structural similarity with testosterone as per our in silico studies was non toxic to the normal mouse peritoneal macrophages as shown in Fig 19. Even after 48h of treatment, cells were viable and showed no abnormalities.
All the thirty synthetic compounds were tested for their growth inhibitory activity on DU145 human prostate cancer cell line. These cells grow in as a monolayer culture, and were maintained in DMEM with 10% FBS. DU145 cells (10⁴ / well) were seeded in 96 well plates; treatments were given with different compounds as described earlier. Thirty analogues of finaestride was analysed for their growth inhibitory activity against human prostate cancer cell line. Few compound tested are showing very little or no growth inhibition and few showed marginal effects on cell growth but toxic to normal macrophages as shown in the Fig 10.
Figure 20: Effects of finasteride analogues on DU145 prostate cancer cells growth in vitro. DU145 cells were exposed to different finasteride analogues and after 48h treatment, cell growth kinetics were monitored by MTT assay.
FZ treatment inhibited growth of human prostate cancer cell lines in culture.

DU145 and PC3 cells were seeded in triplicate and exposed to 1-500 fold excess FZ as described earlier. Results indicated that 80 % inhibition of cell growth after 48 h FZ exposure as shown in the Fig 11. Cell growth was inhibited in a dose dependent manner. Dose dependent relationships describe the changes in the effect on cell growth caused by different concentrations of a small molecule in a given time. Based on this experiment, IC50 values of FZ for DU145 and PC3 cell lines were calculated. The graph shows dose dependent growth inhibition of human prostate cancer cells as shown in Fig 21. It appeared that PC3 cell line was more sensitive to FZ treatment as compared to DU145 cells.

*Figure 21: Dose dependent inhibition in human prostate cancer cell lines following FZ treatment. Normalized data showing the percent inhibition of cell growth. DU145 and PC3 cells were treated with DMSO alone or with varied concentration of FZ dissolved in DMSO for 48 h. The data represent percentage graph.*
Calculation of IC\textsubscript{50} values for DU145 and PC3 cell lines.

IC\textsubscript{50} is the half maximal inhibitory concentration and it is the measure of the effectiveness of a compound in inhibiting biological or biochemical function. This quantitative measure indicates how much of a drug is needed to inhibit a given biological process by half in a given time. The result showed that for DU145 and PC3 cell lines the IC\textsubscript{50} values of FZ were 5.7 \textmu M and 5.5 \textmu M respectively as shown in Fig 22.

$$ Figure 22: \text{IC}_{50} \text{ values were calculated using dose dependent growth inhibition data using curve fit program}$$

FZ inhibited growth of cancer cell lines in a time dependent manner.

Time dependent relationships describe the growth inhibition of cells at a fixed concentration of small molecule in a variable time. To examine the growth inhibitory activity of FZ on DU145 and PC3 cell lines, a time dependent study was performed. Cells were seeded in 96 well plates and were treated with 5 \textmu M FZ and incubated for 72 h at 37 C, cells were harvested and processed for MTT assay. DU145 cells showed 80% growth inhibition where as PC3 cells underwent 90% growth retardation following 72h FZ treatment as shown in Fig 23.
Figure 23: FZ induced growth retardation in a time dependent manner. Control DMSO or FZ (5 µM) treated cell lines were examined for their growth after different time intervals. Mean ± SD, p<0.05.

FZ did not inhibit growth of normal mouse primary epithelial cells in culture

Primary skin epithelial cell culture was established from new born pups. The fibroblast cells were separated by differential trypsinization. Primary epithelial cells (about 95% pure) were plated in triplicate in 96 well plates and treated with FZ (5 µM) for 48 h then processed for the standard MTT assay. The result showed that FZ was nontoxic and had no significant growth inhibitory activity on normal mouse primary cells as shown in Fig 24C. FZ treated cells were observed under phase contrast microscope and found to be unaffected and mentioned normal shape and size and without
any abnormality in nuclear morphology indicating no signs of cell death as shown in Fig 24 B & C.

Figure 24: Mouse primary cells were least affected following FZ treatment A: Phase contrast photomicrograph showing mouse primary cells in culture; black arrows indicate cells and a thin black arrow tissue explants. Photograph was taken with green filter under at 10 X magnification for better contrast. B: Photograph taken under 40 x objective C: The primary mouse cells were treated with 5 μM of FZ in triplicate for 48 h and cell viability was measured by MTT assay.

FZ induced altered nuclear morphology in human proste cancer cell lines, characteristic of apoptosis when observed under Differential Interference Contrast Microscope

Differential Interference Contrast microscopy (DIC) is an optical microscopy illumination technique being used to enhance the contrast of unstained, transparent cells. It works on the principle of interferometry to gain information about the optical density of the cell, because of this the cell image is seen as a three dimensional object. The DU145 and PC3 cells were treated with FZ for 48 h as discussed earlier. Cells were washed with PBS and visualized under DIC phase contrast microscope. A significant change in cell
shape and size was observed as shown in Fig 25. Many cells lost their normal morphology, they were less spread out and with altered nuclear morphology. These features are often found in the apoptotic cells.

Figure 25: Altered morphology of the cells following FZ treatment as observed under DIC. Control and FZ (5uM) treated DU145 and PC3 cells were examined under 10 and 20X objectives. A. untreated and FZ treated-DU145 cells. B. untreated and FZ treated PC3 cells were photographed using Nikon fluorescence microscope under 40 and 100 X objectives.
FZ mediated growth inhibition was due to programmed cell death

Hoechst and Propidium iodide staining was used to differentiate between necrotic and apoptotic cells. Hoechst 33342 is a vital dye that can pass through the plasma membrane of living cells but PI can not. PI binds to DNA only when cell membrane is ruptured. Thus with the help of double staining method we can differentiate between apoptotic and necrotic cells. Human prostate cancer cell lines were seeded at 5000 cells per 30mm dish and treated with FZ for 48 h as described earlier. Live cells were stained with both Hoechst 33342 (10ng/ml) and PI (5 ng/ml) for 3h in the medium. Cells were examined under fluorescent microscope. Control and FZ treated cell nuclei with alter morphology were counted. There were many apoptotic cells with distinct nuclear morphologies like: nuclear condensation, fragmentation and apoptotic bodies as shown in Fig 26. FZ induced a considerable number of apoptotic cells in both PC3 and DU145 cell lines. Results showed 64% and 53 % of apoptotic cell death in DU145 and PC3 cell lines respectively following 48h FZ treatment.
Figure 26: Nuclear morphology of control and FZ treated cells. Hoechst 33342 and Propidium Iodide staining were performed to differentiate between apoptotic and necrotic cells in human prostate cancer cell lines after 48h of FZ treatment. Upper panel: untreated and FZ treated DU145 cells; Lower panel: untreated and FZ treated PC3 cells were photographed using Nikon fluorescence microscope under 40 and 100 X objectives. Bar diagram showing the percentage of apoptotic cells morphology after FZ treatment.

FZ induced apoptosis via mitochondrial pathway.

The mitochondrial permeability transition is an important step in the induction of cellular apoptosis. JC1 is a unique fluorescent cationic dye that stains the mitochondria of healthy normal cells bright red, whereas, in apoptotic cells, the mitochondrial membrane potential collapses, and the JC-1 cannot accumulate within the mitochondria. In these cells, JC-1 remains in the cytoplasm in a green fluorescent monomeric form thus JC1 dye is quite suitable for the probing mitochondria in living cells In order to find out if the molecular pathway of apoptosis elicited by FZ is mediated by mitochondrial pathway, we examined the control and FZ treated cells after JC1 staining. Cells were examined under Nikon fluorescent microscope using appropriate filters (585/590nm and 510/527 nm). J-aggregates formation is associated with a large shift of its absorption and fluorescence maxima to a longer wavelength therefore,Green fluorescence (under blue excitation) or red fluorescence (under green excitation) was detected in mitochondria differing in their membrane potential. However, by use of blue excitation and long pass excitation filter system, yellow fluorescence was detected in region where
both red and green fluorescence coexisted. The result showed that the untreated control cells exhibited as yellow spots indicative of the J-aggregated that represent the intact mitochondrial membrane and no change in the membrane potential but in the FZ treated cells there were a significant number of green fluorescent cells suggesting that JC-1 stain is in the monomer form indicative of the change in the mitochondrial membrane potential. Our result indicated that FZ induced apoptosis was perhaps mediated through mitochondrial dependent pathway of apoptosis as shown in Fig 27.

Figure 27: JC1 staining of DU145 and PC3 cells following FZ treatment. Cells were treated with FZ (5 uM) for 48h and stained with JC-1. Control untreated cells were red and FZ treated cells appeared mostly green as marked with broken arrow.
FZ induced apoptosis and release of Cytochrome c as visualized by Immunofluorescence study.

DU145 and PC3 cells were seeded onto coverslips (2 X 10^4 cells per coverslip), and treated with FZ (5μM) for 48h. The cells were then fixed with freshly prepared 4% Para-formaldehyde for 20 min and permeabilized with 1% Triton X-100 for 45 sec at room temperature. Immunofluorescence staining was performed as described in materials and methods section. The cells were observed under Nikon fluorescence microscope. The result showed that in control untreated cells displayed intense green fluorescent due to the presence of Cytochrome c in the cytoplasm. However, when FZ treated cells were compared with that of control cells there was a significant decrease in Cytochrome c staining indicating loss of Cytochrome c in these cells. Mitochondrial damage is coupled to caspase activation pathways. Result of immunofluorescence studies showed that FZ treatment led to the alteration mitochondrial membrane as a result the FZ treated cell showed reduced green color in the cytoplasmic area as compared to control cells as shown in Fig 28. This was an indication of onset of apoptotic cell death.

Figure 28: Immunofluorescence detection of cytochrome c in PC3 and DU145 cells. DU145 and PC3 cells were treated with FZ (5μM) for 48h, permeabilized and fixed and processed for Immunofluorescence staining as described in material and methods section. The secondary antibody used was tagged with FITC showing green in color and the nucleus was counter stained with propidium iodide showing red in color. Thick arrow showed intact cytochrome c and thin arrow show release cytochrome c.
Cell cycle analysis of DU145 and PC3 cells after 48h FZ treatment

Cells (2 X 10^6) were plated in 90 mm culture dishes and were treated with 5μM FZ for 48 hours. After the treatment, cells were trypsinized and fixed in 70% ethanol overnight at 4°C and stained with propidium iodide (40μg/ml) followed by 15μg/ml RNase A treatment and cells were processed for cell cycle analysis. Phases of cell cycle distribution in control untreated and FZ treated DU145 and PC3 cell lines were examined by flow cytometer. In treated cells sub-G1 population increased from 7 to 41% in DU145 and 10 to 44% in case of PC3 cell lines as shown in Fig 29. Increase in Sub G1 population (M1) indicated that cells were undergoing apoptosis. Moreover there was a marginal increase in M phase population in FZ treated cells as compared to controls.

![Graphs showing cell cycle distribution](image)

**Figure 29: FACS analysis of cell cycle.** FACS analysis was done to determine the cell cycle phases and percent apoptosis. ModFit software program was used to analyze the data. A and B are DU145 cells; C and D represent PC3 cells.
Western blot analysis of SRD5A2 protein in prostate cancer cell lines

To examine whether FZ induces or represses the SRD5A2 gene expression, DU145 and PC3 cells were treated with FZ (5 µM) for 48 h. Total protein was extracted and processed for western blot analysis using SRD5A2 polyclonal antibody. Actin was also taken as a loading control. However, we were unable to detect the 28 KDa SRD5A2 protein in our blot. It is possible that the concentration of the protein was too low in the cells and need to enrich by microsome isolation and ultracentrifugation to pellet the vesicles. We could detect a 42 KDa Actin band as shown in Fig 30.

Figure 30: Western blot analysis to examine SRD5A2 protein in prostate cancer cell lines. DU145 & PC3 were harvested and lysed, and 50 µg protein was electrophoresed and subjected to western blot analysis as described in Material & Methods. Actin protein served as an internal control. SRD5A2 protein was unable to be detected. A; DU145 cells, Lane 1: Protein ladder (in KDa), Lane 2: untreated cells, Lane 3: FZ treated cells. B; PC3 cells, Lane 1: untreated cells, Lane 2: FZ treated cells, Lane 3: Protein ladder (in KDa).

Reduced SRD5A2 mRNA expression in human prostate cancer cell lines alters 48 h FZ treatment.

The DU145 and PC3 cells were plated and treated with FZ for 48 h as described earlier. Then total mRNA was isolated from the cells and converted to cDNA. It was used as template to amplify 315 bp SRD5A2 coding sequences using appropriate PCR primers. PCR product was run on a 2 % agarose gel and photographed and quantified using Image J software as shown in Fig 16. Results indicated that SRD5A2 mRNA level was reduced in FZ treated cells as compared to untreated controls. There was about three-fold reduction in SRD5A2 mRNA in DU145 cell line as shown in the Fig 31A.
and B and two fold in PC3 cell line as shown in the Fig 31 C and D. The 18sRNA amplification was taken as the internal control in the experiments.

Figure 31: SRD5A2 expression in control and FZ treated cells. DU145 and PC3 cells were treated with 5 μM of FZ in DMEM medium for 48h and total RNA was isolated and converted to cDNA. 18S rRNA and SRD5A2 cDNA segments were amplified. A and B DU145 cells and C and D PC3 cells. The bar graph indiciates the realative amount of SRD5A2 after being normalized with 18S rRNA level.
Real time PCR analysis of SRD5A2 experiment after FZ treatment

Expression of SRD5A2 was examined using SYBR Green method of Real time PCR (Eppendorf). Result showed that there was two fold reduction in the expression of SRD5A2 gene expression after 48 h of FZ treatment as shown in Table 10.

Figure 32: Relative quantification graph of SRD5A2 after FZ treatments.

Table 10 # Expression level of SRD5A2 after 48h of FZ treatment

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Tumor susceptibility gene expression (TSG 101) did not change after 48 h FZ treatment as indicated by RT-PCR analysis.

Tumor susceptibility gene 101, also known as TSG101, belongs to the group of apparently inactive homologs of ubiquitin-conjugating enzymes. The protein plays a role in cell growth and differentiation and act as a negative growth regulator. It has been suggested that the expression of this tumor susceptibility gene appears to be important for maintenance of genomic stability and cell cycle regulation. Therefore, an attempt was made to examine the expression of TSG 101 gene after FZ treatment. RT-PCR analysis was performed and 350 bp segment of cDNA. TSG-101 was PCR amplified from control untreated and FZ treated cells as discussed earlier. The PCR products were run on a 2% agarose gel, stained with ethidium bromide and photographed. Gel was quantified using Image J software. The FZ treatment marginally increased the expression of TSG 101 in PC3 cells (1.3 times) as compared to the untreated cells as shown in the Fig 33 C and D. The level of TSG do not alter in DU145 cells as shown in the Fig 33 A and B The 18sRNA PCR product was taken as the internal control of the experiment.
Figure 33: TSG101 mRNA expression in prostate cancer cell lines after FZ treatment. DU145 and PC3 cells were treated with 5 μM of FZ in DMEM medium for the 48h, total RNA was isolated and converted to cDNA and 18S rRNA and TSG101 gene segments were amplified. A and B DU145 cells and C and D PC3 cells. The bar graph indicate the relative amount of TSG101 after being normalized with 18S rRNA level.


The p21 and MDM2 gene are transcriptional regulated by p53. They are major regulator of cell cycle and apoptosis. To examine whether these genes are differentially regulated following FZ. p21 and MDM2 mRNAs were quantified by RT PCR. The DU145 and PC3 cells were treated as before. A 250 bp portion of MDM2 and a 500 bp segment of p21 cDNA were amplified, run on agarose gel and stained with ethidium bromide. These stain gels were examined under gel documentation system (Bio-Rad). The band intensities were quantified using Image J software. FZ treatment for 48h did not change expressions of p21 and MDM2 genes following FZ treatment in both the cell lines as shown in the Fig 34. DU145 cells intense band of p21 in comparison to the PC3 cells.
Figure 34: MDM2 and p21 gene expressions in prostate cancer cell lines after FZ treatment. Agrose gel electrophoresis of p21 and MDM2 PCR products. DU145 and PC3 were treated with 5 μM of FZ in DMEM medium for the 48h and total RNA was isolated and converted to cDNA. 18S rRNA, p21 and MDM2 gene were amplified and photographed. A and B DU145 cells and C and D PC3 cells. The bar graph indicate the relative amount of p21 and MDM2 after being normalized with 18S rRNA level.
Altered expression of pro-apoptotic gene after 48h FZ treatment.

BAK and BAX are genes belonging to the BCL2 gene family. BCL2 family members form oligomers or heterodimers and act as anti- or pro-apoptotic regulators that are involved in a wide variety of cellular activities. Therefore, it was of significant interest to examine whether FZ induced apoptosis is mediated through the BCL2 family of proteins. Therefore, the expressions of BAK and BAX were investigated in FZ treatment. Interestingly, it was observed that BAK expression was downregulated in FZ treated DU145 and PC3 cells as compared to untreated control cells as shown in Fig 35 but on the other hand down regulation BAX expression in DU145 cells as shown in Fig 35 A and B. But in PC3 cells there was an up regulation in the BAX expression.
Figure 35: Expressions of BAK and BAX genes in prostate cancer cell lines, after FZ treatment. We made the cDNAs from the DU145 and PC3 treated with 5 μM of FZ as described earlier with PCR product size 150 bp, 633 bp and 576 bp of 18S rRNA, BAK and BAX genes were amplified in DU145 cells and PC3 cells. Quatitation was done using Image J programme. p > 0.05.

Real time analysis of BAK expression after FZ treatment

Real time PCR analysis of BAK expression was seen after the 48 h of FZ treatment. Result indicated that FZ downregulated BAK expression by four times in PC3 cells as shown in the Table 11.

Figure 36: Relative quantification graph of BAK expression in PC 3 cell line after FZ treatment.
Table 11 # Expression level BAK gene after FZ treatment

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Anti apoptotic genes are down regulated in FZ treated prostate cancer cell lines.

Bcl-xl is a transmembrane molecule in the mitochondria. It is involved in the signal transduction pathway of the FAS-L. It is one of several anti-apoptotic proteins that are members of the Bcl-2 family of proteins. After FZ treatment, total mRNA was isolated from the cells and converted into cDNA. A 750 bp Bcl-xl and 600 bp BCl2 PCR products were amplified using appropriate primers and were run on 2% agarose gel and quantified. Apparently, FZ treatment had no effect on Bcl-xl expression these cells lines. However, in DU145 cell line there was a twofold reduction in BCl2 expression, one fold reduction in PC3 cells was noticed (Fig 29).
Figure 37: RT-PCR analysis of BCI2 & Bcl-xl genes. cDNAs were prepared from control untreated and FZ treated cells and 18S rRNA, Bcl-xl & Bcl2 PCR products were quantified. A & B DU145 and C & D PC3 cells. p value ≥ 0.05.
FZ induced apoptotic cells showed characteristic DNA fragmentation.

Nuclear DNA fragmentation is characteristic feature of programmed cell death. In this process chromatin, DNA is cleaved in 180 bp fragment and its multiples. Genomic DNA from the control untreated and 48h FZ treated cells were isolated as described in materials and methods. Total 5 μg of genomic DNA was run on TBE gel electrophoresis and stained with ethidium bromide. The result showed that FZ induced significant DNA fragmentation during apoptosis. A characteristic DNA ladder was visible in DNA sample isolated from FZ treated cells where as control DNA did not show any DNA fragmentation. These results confirm further that FZ induced cell death is mediated by apoptosis but not due to necrosis as shown in the Fig 30.

**Figure 38:** DNA fragmentation was observed in cells treated with FZ. The nuclear DNA of control and FZ treated DU145 and PC3 cells were isolated and separated and run on 1.5 % agarose gel and stained with ethidium bromide and photographed significant DNA fragmentation was observed in 48 h FZ treatment cells as compared to control untreated cells. Lane 1, control; Lane 2, FZ treated.

FZ induced apoptotic cells showed single strand breaks detected by TUNEL assay

Extensive DNA degradation is a characteristic event that occurs in the late stages of apoptosis. Cleavage of the DNA may yield double-stranded, LMW DNA fragments (mono- and oligonucleosomes) as well as single strand
breaks ("nicks") in HMW-DNA. Those DNA strand breaks can be detected by enzymatic labeling of the free 3'-OH termini with modified nucleotides (X-dUTP, X = biotin, DIG or fluorescein). Suitable labeling enzymes include DNA polymerase (nick translation) and terminal deoxynucleotidyl transferase (end labeling). Terminal deoxynucleotidyl transferase (TdT) is able to label blunt ends of double stranded DNA breaks independent of a template. The end-labeling method has also been termed TUNEL (TdT-mediated X-dUTP Nick End Labeling). In our result it is clearly seen that FZ induce strands break in DNA as shown in Fig 39.

**Figure 39: TUNEL assay after FZ treatment.** Apoptotic cells were identified by TUNEL staining. Control untreated cells after 48 h of treatment with FZ with untreated control. Apoptotic cells appeared dark after staining. In contrast, no increased staining was seen untreated control cells. A DU145 and B PC3.

**Antitumor activity of FZ in vitro.**

Colony formation assay is based on the ability of a single cancer cell to grow into a colony. Thus, every cell in the population may have the ability to go unlimited division. Therefore, Colony formation assay is a way to determine the cytotoxic effect of a drug on cells in vitro it provides enough time to the cells to recover, repair the damage and proliferate, or to undergo apoptosis if the damage is irreversible. Thus in vitro tumor clonogenic assay has proven predictive value with the chemosensitivity testing of standard and experimental anticancer drugs. To test the anti-tumorigenesis effect of FZ on DU145 and PC3 cell lines, colony formation assay was carried out. Fixed number of DU145 and PC3 cells were seeded in 35 mm plates and treated with 5 μM FZ for 24 hours. Next day, cells were washed and replenished with
fresh medium. Cells were allowed to grow for two weeks, and then they were fixed and stained with crystal violet. Both the cell lines formed distinct colonies, more than 50 cells together, were considered as a colony. Untreated control cells formed much larger colonies as compared to FZ treated cells. Moreover, the overall colony number was less in FZ treated cells. Thus, it appears that FZ has antitumor property. There was a threefold reduction in colony forming ability in DU145 and PC3 cells lines after FZ treatment as compared to the respective untreated controls as shown in Fig 40.

Figure 40: FZ inhibits colony forming ability of DU145 and PC3 cells in culture. DU145 and PC3 Cells were treated with FZ for 24 h and processed for colony formation assay, quantitation of colonies in control and FZ-treated cells were done by manual counting. The colonies are indicated like dots. Control panel showed more colonies seen as blue in color. FZ treated cells showed fewer and much smaller number of colonies.

Oral feeding of FZ had no toxicity in mice

There is no toxicity of FZ in animals. Mice were fed with different concentrations of FZ without any adverse effect. A dose escalation studies was performed and it was found that 200 mg/Kg body weight FZ dose every day for a month had no effect on diet consumption, change in body weight, posture & behavioral changes during the course of study.
Oral feeding of FZ inhibits DU145 tumor xenograft growth in nu/nu mice.

The growth inhibitory effects of FZ on proliferation of tumor cells in vitro promoted us to investigate the antitumor activity of this compound in in-vivo nu/nu mouse model. Tumors were established in these mice by subcutaneous injection of $5 \times 10^6$ DU145 cells. After the mice had established tumors (2-4 mm in diameter), they were fed orally with FZ dissolved in olive oil (160mg/kg body weight of mouse) every other day for two weeks, whereas control animals received olive oil only, five animals in each group. FZ treated mice showed no toxicity and were all healthier so as control mice during two weeks treatment. Significant decrease in tumor volume was observed in animals fed with FZ as compared to mock control as shown in Fig 41. DU145 tumors were then excised and photographed as shown in the Fig 41 A & B, and weighted. There was a marked difference in tumor weight between the FZ-treated animal versus control animals as shown in the Fig 43. Further, the tumor vascularity in vivo was quantitated between control and FZ treated mice using a hemoglobin assay. Results showed that there was about 50 % reduction in hemoglobin content/milligram in tumors obtained from FZ treated mice, as compared with control mice as shown in the Fig 44. Suggesting that FZ inhibited the angiogenesis in tumor in vivo. Histological studies of the tumor sections using staining indicated that a number of cells in the FZ treated mouse showed many fragmented tumor cell nuclei indicating that perhaps FZ inhibited the in vivo tumor growth by inducing apoptosis in these cell as shown in Fig 45.

Figure 41: Reduced tumor growth in mice after oral administration of FZ. A; Photographs showing mice with tumor burden in control and two weeks after FZ treatment. B; Tumor volumes of untreated and FZ treated mice after two weeks.
Figure 42: FZ fed mice had smaller tumor size as compared to control mice. Tumor volumes were calculated for the untreated and FZ treated tumor individually. Changes in the tumor volume were plotted against treatment time. The statistical significance of the of the expression were determined by unpaired student t test having $p$ value $\leq 0.01$.

Figure 43: Tumor weights were estimated. Bar graph showing tumor weight of control and FZ fed animals $p$ value $\leq 0.05$. 
Figure 44: Hemoglobin content of FZ treated animals was less than that of control group. Graphical representation of hemoglobin content in tumor tissues obtained from untreated and FZ treated animals. The statistical significance in hemoglobin content were determined by unpaired student t test ($p < 0.05$).

Figure 45: Hematoxyline and Eosin staining of DU145 xenograft tumor sections. Histological analysis of DU145 xenograft tumors after hematoxylin and eosin staining, control: untreated mice showed normal histoarchitecture (upper panel); FZ fed mice showed significant increase in the apoptotic nuclei in the tumor as shown in lower panel. Sections were examined under 4X and 10X objective.
Real time PCR analysis of von Willebrand factor (VWF) expression in tumor tissues

Von Willebrand factor (VWF) is a multimeric plasma glycoprotein that plays a critical role in primary hemostasis. In metastatic cancer, tumor cells interact with platelets and the vessel wall to extravagate from the circulation. A number of potential receptors for VWF have been identified on tumor cells such as glycoprotein and direct interactions between VWF and tumor cells have been reported. VWF plays a role in tumor metastasis, independently of its role in hemostasis. However, we have seen a significant difference in the expression of VWF level after FZ treatment as shown in Fig 46.

Figure 46: Real time PCR product of von Willebrand factor amplified from untreated control and FZ fed nude mice. A; Photograph showing, Lane 1 to 3; Untreated Beta Actin, Lane 4 to 6 FZ treated Beta Actin, Lane 7 to 9; Untreated vWF PCR product & Lane 10 to 12 FZ treated vWF PCR product. B; The bar graph indicates the relative amount of von Willebrand factor after being normalized with Actin level.
Real time PCR analysis of MMP9 gene expression in the tumor

Tissue remodeling involving the degradation of the extracellular matrix occurs not only in normal development but also in pathological conditions such as rheumatoid arthritis and tumor invasion and metastasis. Metalloproteinases (MMPs) play an important role in this process, inducing a variety of biological effects including growth, morphogenesis, apoptosis, tissue destruction, and cancer. MMP-9, whose expression has been implicated in atherosclerosis, inflammation, rheumatoid arthritis, and tumor invasion. In our result of real time PCR analysis of MMP 9 gene it showed no significant difference after FZ treatments as shown in Fig 39.

![Figure 47 : MMP 9 gene amplified from untreated control and FZ fed nude mice. A; Photographs showing, Lane 1 to 3; Untreated Beta Actin, Lane 4 to 6 FZ treated Beta Actin, Lane 7 to 9; Untreated MMP 9 gene PCR product & Lane 10 to 12 FZ treated MMP 9 gene PCR product. B; The bar graph indicates the relative amount of MMP 9 factor after being normalized with Actin level.](image-url)
Real time PCR analysis of CD 31 gene in tumor of nude mice

CD31 recognizes a 100kDA glycoprotein in endothelial cells and 130kD in platelets. It reacts weakly with mantle zone B-cells, peripheral T-cells, and neutrophils. CD31 can detect vascular endothelium associated antigen and has been used as a marker for benign and malignant human vascular disorders, myeloid leukemia infiltrates and megakaryocytes in normal bone marrow. When compared to Factor VIII and CD34 antibodies, studies have shown CD31 to be a superior marker for angiogenesis. CD31 has been used to measure angiogenesis. In our CD31 expression is downregulated, which indicate the decrease in the angiogenesis as shown in Fig 48.

Figure 48: Real time PCR product of CD31 gene amplified from untreated control and FZ fed nude mice. A: Photograph showing, Lane 1 to 3; Untreated Beta Actin, Lane 4 to 6 FZ treated Beta Actin, Lane 7 to 9; Untreated CD31 gene PCR product & Lane 10 to 12 FZ treated CD31 gene PCR product. B; The bar graph indicates the relative amount of CD 31 after being normalized with Actin level.
Real time analysis of Vascular endothelial growth factor (VEGF) gene expression in tumors of nude mice

Vascular endothelial growth factor, or VEGF, is the key signal used by oxygen-hungry cells to promote growth of blood vessels. It binds to specialized receptors on the surfaces of endothelial cells and directs them to build new vessels. After receiving this message, the cells build specialized proteases to break through the basal lamina, and migrate into the oxygen-starved region. Once there, the cells multiply and form into tubes, creating a new path for blood to flow. In tumor formation there are constantly process of formation of new vessels. In our result, VEGF expression don not showed any difference as shown in Fig 49.

Figure 49: Real time PCR product of Vascular endothelial growth factor (VEGF) gene was amplified from untreated control and FZ fed nude mice. Untreated group : Lane 1 to 3; beta actin, Lane 7 to 9. VEGF : FZ treated group : Lane 4 to 6, beta actin. Lane 10 to 12, VGEF.
**Estimation of total serum testosterone level in mice after FZ treatment**

In the view of my earlier experimental data, it was interesting to examine the level of serum testosterone in mouse after FZ treatment. Normal male balb/c mice were fed orally with FZ dissolved in olive oil for five days. *In vivo* interaction of FZ with testosterone was studied after five days of oral dosing. Mice were fed with FZ (1 mg/kg body weight) and positive control group of mice were fed with Finaestride (1 mg/kg body weight) for five day. After the treatment, mice blood samples were collected by puncturing the retro-orbital venous sinus using sterilized glass capillaries. The collected blood was processed for the isolation of serum. The approximately 300 µl of serum was used to estimate the total testosterone level. The total serum level was estimated by colorimetric based Roche kit. Total testosterone concentration was calculated in ng/ml and plotted in the bar diagram. When the testosterone level of the finasteride and FZ group was compared with the untreated control group result showed that there was four-fold reduction in the total testosterone level as shown in the Fig 50.

![Figure 50: Total testosterone level after five days of FZ treatment.](image_url)

**Figure 50:** Total testosterone level after five days of FZ treatment. Total testosterone level was estimated in the blood serum of mice after five days of FZ treatments. Dusteride was a known inhibitor of testosterone taken as a positive control. The olive oil was taken as a vehicle control.
Discussion

Prostate cancer is a dreadful cancer that kills second highest number of men worldwide. One in 10 men in the developed world will present with prostate cancer and in an ageing population, developing strategies for its chemoprevention or treatment is of significance. For decades, androgen ablation has remained the frontline treatment for prostate cancer and thus become surgically inoperable. Orchidectomy or drug induced reduction of tumor growth promoting effects in the prostate is used. However, resistance often develops within a few months to years and androgen-insensitive tumors develop. In past years, there has been an increasing focus on chemoprevention with agents such as finasteride being employed to reduce the risk of developing prostate cancer. Significantly, such chemoprevention strategies are also based on 5a-reductase inhibition thus reducing intraprostatic dihydrotestosterone levels. A histological change in the prostate, (BPH) is a normal part of aging. However, BPH disease, defined here as a life-altering urinary condition caused by BPH requiring prompt medical intervention, is a serious medical disorder associated with major complications, surgical intervention, and severe lifestyle interference. BPH disease is preventable. Men at greatest risk of BPH disease can be identified using prostate-specific antigen (PSA) level higher than 1.5 ng/mL as a surrogate marker for an enlarged prostate and 5α-reductase inhibitors (5ARIs) reduce the primary androgen responsible for prostate growth (dihydrotestosterone), shrink the prostate. As result of the past studies, we have also screened a library of chemical analogues of finasteride for their anti-cancer activity against human prostate cancer cells. While DHT seems to be the main androgen stimulating and maintaining the growth of prostate, it is not clear whether the androgen dependence of prostate cancer is also mainly related to DHT. This suspicion was first raised by the findings of Brooks [116], who applied Finasteride to the Copenhagen rat Dunning prostatic adenocarcinoma model R-3327. By using the androgen-dependent H line, the usual effects were seen on the animals own ventral prostate. No effect on the growth of the Dunning-H tumour could, however, be found. Castration and diethylstilboestrol in the control group did suppress significantly the growth of
the Dunning tumours. These findings were confirmed by Lamb [117]. They found that the Dunning R-3327-G but not the H subline was inhibited significantly by the compound SK & F-105657, a non-competitive 5αR inhibitor. While the extensive study of Lamb explains some of the negative results obtained with Finasteride and also with SK&F-105657 in some Dunning tumour cell lines and in the human cell line PC-82, the mechanism of failure of response for some cell lines to Finasteride. In our studies, we have screened thirty small molecules or effect of using human prostate cancer cell line as in vitro model system and nu/nu mice model for in vivo tumorigenesis model as shown in Fig 41 and their effect on normal primary cell culture and macrophages cells of mouse as shown in Fig 24 & 19. As result showed, most of these compounds were toxic to the normal cells hence were not further studied. Few compounds were showing growth inhibitory activity against prostate cancer cell lines but were very toxic to the normal cells also. In this study, we have first shown that Methyl 5-[phenylthio]-2 benzimidazole carbamate (FZ) exerts a significant degree of growth inhibition on prostate carcinoma cell lines. These cell lines are very good models in which to study the effects and mechanisms of various drugs because they represent the highly aggressive nature of metastatic human prostate. We used DU145 and PC-3, which are human hormone-independent prostatic carcinoma cell lines. IC50 value of FZ was calculated for both cells which slightly different for these cell lines 5.7 and 5.5 μM for DU145 and PC3 respectively. At these concentrations a considerable tumor cell killing was noticed. Apoptotic cell death appeared to be the main phenomenon resulting in significant tumor cell death. Most importantly, it showed no toxicity to the normal cells in culture or in vivo in the mice as such. FZ is a benzimidazoles group of compound that showed success in their selective toxicity against helminthes. These groups of small molecules have been reported to have poor systemic absorption after oral administration in vivo. The observed safety of the benzimidazoles as anthelmintics may also be unrelated to BZ-tubulin binding but rather may be attributable to differences in the metabolic or detoxification pathways as suggested by Nare et al [118]. For example, rapid, extensive metabolism of BZs into less toxic metabolites (e.g. sulfoxides and sulfones) by the hepatic
microsomal enzymes [119, 120] may account for some of the lack of host toxicity. Parasites, on the other hand, lack these metabolic pathways and are killed by benzimidazole. FZ was examined for the inhibition of SRD5A2 as shown in Fig 18. For the past many years researchers discovered large number of small molecules against prostate cancer [116]. In our result, there was about three-fold reduction in SRD5A2 mRNA in both the prostate cancer cells. SRD5A2 inhibition in these cell leads them to the change in the morphology a observed by differential interference contrast microcopy as shown in Fig 25 there significant in the cell shape and size. These changes in the morphology was confirmed by the Hoechst 33342 and propidium iodide staining as shown in the Fig 26. It was observed that there was considerable numbers of apoptotic cell death in DU145 and PC3 cell lines after 48h of treatments. Further, apoptotic cell death was examined of the mechanism or pathways involved in FZ mediated cell death. FZ induced the release of cytochrome c as examined by JC 1 as shown in the Fig 28 as a significant no of cell showed positively stained for cytochrome c, which indicated the FZ induced cell death is mitochondrial dependent. In previous reports, It was that androgen plays a significant role in the inhibition of prostate cancer cell growth. In the absence of androgens, prostatic epithelial cells undergo apoptotic cell death, an active process that is normally inhibited by the presence of androgens. The effect of androgen withdrawal in humans has best been studied in cases of BPH and Prostate cancer. In both instances, the prostate diminishes in volume. The volume reduction after surgical castration or treatment with an agonist or a steroidal anti-androgen amounts to about 30% and reaches a maximum after 3 months [121-123]. The data on the effects of androgen ablation in BPH have recently been summarized by [124]. The study by Peters and Walsh, which included the morphometric evaluation of biopsy specimens, showed that the epithelial and stromal compartments are about equally involved in the process of shrinkage. Unfortunately, the effects of prostatic shrinkage induced by androgen withdrawal on functional parameters related to micro-nutrition are minimal [125]. Clearly, in the adult human situation, the effects of androgen withdrawal are considerably less pronounced than the absence of androgens during embryonic development. The prostate harboring prostate cancer responds to androgen withdrawal in a
more pronounced way than BPH. The volume reduction after castration has been shown by Sneller et al. [126] to average 30-50%. FZ was further studied whether it induced any change in cell cycle. In our result, it showed that FZ induce sub G1 population to 40% that indicated that cell under goes apoptosis. The effect of FZ as an antitumor agent has never been tested before. With that in mind, one of our most encouraging findings was that FZ inhibited tumor cell growth both in vitro and in the human xenografts, in our result FZ induces the tumor growth in vivo as indicated in Fig 41 In conclusion, it can be said that FZ is a potent anti cancer agent used in prostate cancers.