THE EFFECT OF FINASTERIDE AND DUTASTERIDE ON THE GROWTH OF WPE1-
NA22 PROSTATE CANCER XENOGRAFTS IN NUDE MICE

by

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Abstract

5α-reductase 1 (5αR1) and 5α-reductase 2 (5αR2) convert testosterone into the more potent androgen, dihydrotestosterone (DHT), that is responsible for regulating prostate growth and proliferation. 5αR2 is the main isoenzyme in normal prostate tissue, however prostate tumors have increased 5αR1 and decreased or unchanged 5αR2 expression. Previously, finasteride (5αR2 inhibitor) treatment begun 3 weeks after tumor implantation had no effect on Dunning R3327-H rat prostate tumor growth. We believe the tumor compensated for finasteride treatment by increasing tumor 5αR1 activity to produce dihydrotestosterone to stimulate its growth. We hypothesize that finasteride treatment would not significantly alter tumor growth even if begun before tumor implantation, while dutasteride (dual 5αR1 & 5αR2 inhibitor) treatment would decrease tumor growth regardless if treatment is begun before or after tumor implantation. Sixty, 8-week old male nude mice were randomized to Control, Pre-Finasteride, Post-Finasteride, Pre-Dutasteride and Post-Dutasteride diet groups (all diets contained 83.3 mg drug/kg diet). Pre groups began their treatment diets 1-2 weeks prior to tumor implantation, while post groups began their treatment diets 3 weeks after tumor implantation. Tumors were implanted by subcutaneous injection of 1 x 10^5 WPE1-NA22 human prostate cancer cells in Matrigel™ and allowed to grow for 22 weeks. Tumor areas, body weights, and feed intakes were measured weekly. At study conclusion, prostate and seminal vesicle weights were significantly decreased in all treatment groups versus the control. Dutasteride intake also significantly reduced seminal vesicle weights compared to finasteride intake. There were no significant differences in final tumor areas or tumor weights between groups, likely due to poor tumor growth. In follow-up studies, proliferation of WPE1-NA22 prostate cancer cells, and its parent line RWPE-1 prostate

epithelial cells, were unaltered by treatment with testosterone, DHT, or the synthetic androgen mibolerone, suggesting that these cell lines are not androgen-sensitive. Thus, the lack of response to androgen treatment by WPE1-NA22 prostate cancer cells may explain the inadequate tumor growth observed.

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Dedication

I dedicate this work to my dearest mum, Mrs. Faustina Felicity Opoku-Acheampong who has been a very strong pillar behind my education. Mum, you have been a blessing to me and I love you very much! I cannot leave out my beautiful and lovely fiancée Audrey Anima Bampoe for proof-reading and support. God richly bless you. Last but not the least; I dedicate it to my wonderful siblings Dora Opoku-Acheampong and Alex Okae-Acheampong. Thank you all!

Chapter 1 - Introduction

Prostate cancer is the second leading cause of cancer related deaths and was estimated to account for one out of every four newly diagnosed cancers in American men in 2010 [1]. Within the prostate, the main circulating androgen testosterone is converted by the enzymes 5α-reductase 1 and 2 into the more potent dihydrotestosterone (DHT), which binds with up to 10-fold higher affinity to the androgen receptor (AR) than testosterone [2,3]. The binding of DHT to androgen receptor causes gene transcription of androgen-regulated genes and promotes cellular proliferation in androgen-dependent cancerous tissues [4]. At initial stages of prostate cancer, most cancers rely on androgens for growth, and thus are referred to as androgen-dependent [5]. Inhibiting androgen production and/or blocking its action have served as means to combat prostate cancer [6].

5α-reductase 1 is the major isoenzyme in human liver and non genital skin, while 5α-reductase 2 is the major isoenzyme in prostate, epididymis, seminal vesicles and genital skin. [7]. Most studies report increased levels of 5α-reductase 1 and decreased levels of 5α-reductase 2 in prostate cancer [8-12], others observed increased 5α-reductase 1 mRNA expression and no significant changes in 5α-reductase 2 mRNA expression in prostate cancer versus normal cancer [13], increased expression of both isoenzymes in prostate cancer [6], or loss of expression of both isoenzymes in metastatic prostate cancer [14]. There are two 5α-reductase inhibitors, finasteride (5α-reducatse 2 inhibitor) and dutasteride (5α-reductase 1 and 2 inhibitor), commonly used to treat benign prostatic hyperplasia (BPH) [15]. There is also the potential that these 5α-reductase inhibitors could be used to prevent or treat prostate cancer by reducing DHT levels [16].

To this end, the Prostate Cancer Prevention Trial (PCPT) found that finasteride decreased prostate cancer prevalence by 24.8% [17]. Similarly the Reduction by Dutasteride of Prostate Events (REDUCE) trial found a 23% reduction in prostate cancer incidence with dutasteride administration [18]. In animal models, finasteride did not inhibit growth of the Dunning R-3327H rat prostate tumors while dutasteride did [19]. In nude mice bearing LNCaP human prostate cancer xenografts, both finasteride and dutasteride reduced tumor growth, although dutasteride was more effective at an equimolar dose [19]. Similarly in another rat study, finasteride significantly decreased weights of the androgen-sensitive tissues, seminal vesicles and prostate, but did not decrease Dunning R-3327H tumor growth [20].

In these animal studies, finasteride and dutasteride administration began after tumors were already established so it is possible that if finasteride was administered before tumor implantation, it might significantly reduce tumor growth. On the other hand, regardless of when finasteride treatment is begun, prostate cancer cells may compensate for 5α-reductase 2 inhibition by increasing 5α-redutase 1 expression and/or activity. Thus, the dual inhibitory effect of dutasteride may offer an advantage over finasteride. We examined the effect of finasteride and dutasteride diets begun (1 week) before or (3 weeks) after subcutaneous injection of WPE1-NA22 human prostate cancer cells into the rear flanks of male nude mice. We chose to use WPE1-NA22 prostate cancer xenografts because these are human cancer cells that can be cultured *in vitro*, form tumors that are not invasive and have growth rates and pathology similar to the Dunning R-3327H tumor [21,22].

Chapter 2 - Literature Review

Prostate Cancer

Prostate cancer accounts for one out of every four newly diagnosed cancers in American men with an estimated 217,730 new cases in 2010. Early detection is possible through prostate specific antigen (PSA) testing although the latent nature and limited clinical symptoms associated with the disease can hinder its detection. Factors such as age, race, socioeconomic status and genetic predisposition play a significant role in prostate cancer occurrence. Mortality is variable with some men living for many years without showing any clinical symptoms while others die a short time after diagnosis [1].

Prostate Intraepithelial Neoplasia (PIN) and Benign Prostatic Hyperplasia (BPH)

Two other common prostate conditions are prostate intraepithelial neoplasia (PIN) and benign prostatic hyperplasia (BPH). PIN is a microscopic lesion in the prostate believed to be a pre-cancerous condition [23]. Over 50% of men aged 60 or over are believed to suffer from BPH [24], which is caused by increased proliferation of prostate stromal and epithelial cells [25]. BPH is clinically distinguished by the progressive development of lower urinary tract symptoms [26] as a result of the compression of the urethra caused by enlargement of the prostate [24]. Symptoms include nocturia, incomplete emptying, urinary hesitancy and weak stream [26].

Prostate Specific Antigen, Gleason Grading and Tumor, Lymph Node and Metastasis (TNM) Staging

The prostate epithelium produces prostate specific antigen (PSA), a glycoprotein that since 1987 has been used as a biomarker for prostate cancer screening [27]. Although men with serum PSA levels greater than 4 ng/ml are regarded as being at increased risk, this threshold is

controversial because men with PSA levels below this threshold also develop prostate cancer [28,29]. Typically, men with prostatic diseases including prostate cancer have higher serum PSA levels because of increased production coupled with the architectural distortions within the prostate [27]. PSA is found free in circulation as well as bound to the serum proteins α_1-antichymotrypsin and α_2-macroglobulin [30]. Total serum PSA can be increased in other prostatic conditions making it a less sensitive and specific tumor biomarker [31]. Subsequently, the ratio of free PSA to total PSA has been reported to be better in distinguishing prostate cancer from BPH [32]. Other parameters have also been introduced to improve PSA as a biomarker of prostate cancer such as PSA density [33], serial PSA measurements [28], age and race specific reference ranges [34] and prostate specific velocity (rate of change of PSA over time) [35].

Gleason grading is a prostate cancer measure that uses five histologic grade patterns ranging from 1 to 5. A histologic score is obtained by the summation of the primary grade pattern and the secondary grade pattern to give a range from 2 to 10. In situations with only one grade pattern present, it is multiplied by 2 to give the histologic score [36]. The tumor, lymph node and metastasis (TNM) is another staging method of prostate cancer. The T describes the size of the tumor, N describes the regional lymph nodes, and M describes distant metastasis. A range of numbers is used to classify the parameters T, N and M (eg. T1: is not palpable or visible, T2: confined within the prostate and T3: through prostate capsule; NX: cannot evaluate regional lymph nodes, N0: no spread to regional lymph nodes N1: spread to regional lymph nodes; MX: cannot evaluate distant metastasis, M0: no distant metastasis; M1: distant metastasis) [37,38,39].

Androgens

Androgens are male steroid hormones that control the development and differentiation of the male reproductive system [40]. Within the prostate, the main circulating androgen testosterone is converted by the enzymes 5α-reductase 1 and 2 into dihydrotestosterone (DHT), the more potent androgen required for normal growth and development of the prostate [41-43]. Although both testosterone and DHT bind to the androgen receptor, DHT binds with a 10-fold higher affinity [2,3]. The binding of DHT to the androgen receptor causes the transcription of androgen-regulated genes and cellular proliferation in androgen-sensitive tissues [4]. At initial stages of prostate cancer, most are referred to as androgen-dependent. However, prostate cancer often becomes androgen-independent when patients undergo androgen ablation treatment for an extended period. At this stage, prostate cancer does not depend on androgens for growth, meaning it can grow even under androgen-depleted conditions. The mechanism through which prostate cancer becomes androgen-independent is not known but could be a result of genetic change in prostate cancer cells, androgen receptor (AR) hypersensitivity, ligand-independent AR activation, AR bypass, and change of AR specificity by mutation [5].

5α-Reductase Enzymes and 5α-Reductase Inhibitors

5α-reductase 1 is the major isoenzyme in human liver and non genital skin while 5α-reductase 2 is the major isoenzyme in prostate, epididymis, seminal vesicles and genital skin [7]. 5α-reductase 1 and 2 enzymes are pH dependent and are maximally active at alkaline and acidic pH, respectively [44]. Most literature report increased levels of 5α-reductase 1 and decreased levels of 5α-reductase 2 in prostate cancer [8-12], others observed increased 5α-reductase 1 mRNA expression and no significant changes in 5α-reductase 2 mRNA expression in prostate cancer versus normal cancer [13], increased expression of both isoenzymes in prostate cancer

[6], or loss of expression of both isoenzymes in metastatic prostate cancer [14]. Recently, 5α-reductase 3 has been identified and found to be overexpressed in hormone-refractory prostate cancer cells and tissues [45].

There are two inhibitors of 5α-reductase 1 and 2 commonly used to treat BPH, finasteride (trade name Proscar[®]) and dutasteride (trade name Avodart[®]). In addition finasteride (trade name Propecia[®]) is also used for treating male pattern baldness [17,46]. They are both azasteroids meaning they contain heterocyclic nitrogen rings [7]. A structural modification in dutasteride increases its serum half-life and inhibition potency [19]. Finasteride competitively inhibits 5α-reductase 2 activity and reduces serum DHT levels by approximately 70%, while dutasteride competitively inhibits both 5α-reductase 1 and 2 isoenzymes and decreases serum DHT by greater than 90% [47,48]. This dual inhibition may offer an advantage over finasteride and is approximately 60 times more potent than finasteride in reducing 5α-reductase activity [49,50].

Cell Lines for Prostate Cancer Research

The two types of prostate cell culture options are primary or immortalized cells. Primary cell cultures are derived directly from tumors and have the advantage of being more representative of their original tissue. The disadvantages however are the limited access, finite lifespan and specific culturing techniques for maintaining these cells. Immortalized cell lines are derived from normal or cancer tissues and they offer the advantage of having an unlimited lifespan. However, the disadvantage of these lines is that they are not as representative of the original tissues [51,52].

Extremely poor growth of human prostate tumor tissue in culture and/or in nude mice has been, and continues to be, a major challenge for selecting a prostate cancer cell line. The poor growth of human prostate tissue in culture may be explained by the fact that total and free

testosterone levels in 10% fetal calf serum (commonly used in media for *in vitro* culture of cells) are more than 100-fold lower than is found in serum from intact adult human males. Even more surprising is that total serum testosterone levels in fetal bovine serum are slightly lower than serum testosterone levels from castrated adult human males [53].

The choice of a cell line for prostate cancer research depends on a number of factors such as androgen-sensitivity, doubling time, and tumor forming potential in immunocompromised mice. The three most widely used immortalized prostate cancer cell lines are LNCaP, DU-145, and PC-3 cells, which are all derived from metastasized human prostate cancer [54]. LNCaP cells were derived from lymph node metastasis and are androgen-sensitive meaning androgens stimulate their growth [55-57]. DU-145 cells were isolated from brain metastasis while PC-3 cells were derived from bone metastasis; both lines are androgen-insensitive [58,59]. Over the years, other cell lines have also been developed such as the RWPE-1 prostate epithelial cells derived from the peripheral zone of a histologically normal adult human prostate and immortalized using human papilloma virus-18 (HPV-18) [60]. To create cancerous cells, RWPE-1 cells were transformed using the carcinogen *N*-methyl-*N*-nitrosourea and subcutaneously injected into nude mice to form tumors. Second generation tumors were then cultured, progressively, to create the WPE1-NA22, WPE1-NB14, WPE1-NB11 and WPE1-NB26 cell lines [22]. Another cell line, the PC-346C cells are human, androgen-sensitive cells derived from the transurethral resection of a primary prostate tumor [61]. Some common cell lines and their individual characteristics are listed below.

Table 2.1 Characteristics of Common prostate cell lines [22,55-60]

Cell line	Origin	Androgen sensitive /dependent /independent	Tumor forming potential
DU-145	Brain	AS	Yes
LNCaP	Lymph node	AS	Yes
PC-3	Bone	AI	
PC-346C	Prostate	AS/AI	Yes
WPE1-NA22	Prostate	AS	Yes
RWPE-1	Prostate	AS/AI	No

Spontaneous, Carcinogen-Induced, and Hormone-Induced Models of Prostate Cancer

There are a limited number of laboratory animal models that develop spontaneous prostate cancer. Dogs and ACI Seg rats are the only animals that develop prostate cancer spontaneously [62,63]. Similarities between human and canine prostate cancer include late age of onset, metastatic propensity and androgen-independence associated with advanced stages of the disease [62]. Regardless of these similarities, the dog is not commonly used for prostate cancer research.

ACI Seg rats spontaneously develop prostate cancer in 35-40% of cases by 30 months with no significant stromal inflammation. Carcinogen-induced models include the Fischer F344 rat, which develop tumors in the seminal vesicles, anterior, ventral, and dorsolateral lobes of the prostate after administration of the carcinogen 3,2'-dimethyl-4-aminobiphenyl (DMAB) and testosterone propionate (TP) [64]. *N*-nitroso-*N*-methylurea (NMU) is another carcinogen that is

capable of chemically inducing cancer in this model [65]. The advantage of the Fischer F344 model is their long life span and androgen-dependent tumors; however their inability to develop tumor in bones limit their usability for some metastatic studies. The Noble rat when treated with testosterone and estradiol has 100% incidence of prostate cancer in the dorsolateral region although there is limited metastasis [64].

Xenografts and Transplantable Tumor Models

Nude mouse lack a functional thymus, as a result they can accept subcutaneous grafts of human tumor or cancer cells. To increase the tumor take rate, cancer cells are often mixed with Matrigel™ before injection. In prostate cancer xenograft models, prostate cancer cells are subcutaneously injected into flanks or shoulders, orthotopically injected into the prostate, or implanted into the sub-renal capsule of immunocompromised animal models [66]. LNCaP and PC-346 are human androgen-sensitive xenografts that have been used for studying the progression of prostate cancer from androgen-dependent to androgen-independent [67,68]. CWR22 cells were developed from a primary prostate cancer and are also ideal for studying the progression of prostate cancer from androgen-dependence to androgen-independence [69]. The WPE1-NA22, WPE1-NB14, WPE1-NB11 and WPE1-NB26 prostate cancer cell lines, derived from RWPE-1 prostate epithelial cells, can be used to study carcinogenesis, progression, intervention and chemoprevention. The WPE1-NA22 and WPE1-NB14 are both non-invasive while WPE1-NB11 and WPE1-NB26 are slightly and highly invasive, respectively. The WPE1-NA22 xenografts have the slowest doubling times and form well-differentiated tumors [22]. The Dunning R3327-H transplantable tumor was derived from a spontaneous tumor in a Copenhagen rat. It is slow-growing, non-metastatic, androgen-sensitive tumor that responds to dietary interventions [21,70].

Transgenic Prostate Cancer Models

Transgenic mouse models have been developed that mimic the complexity of prostate carcinogenesis. Genes such as the rat probasin and human prostate specific antigen (PSA) are introduced into the germline of animal models to create stable transgenic models that can be propagated [71]. Two variants of the rat probasin promoter have been used to develop two transgenic models; the transgenic adenocarcinoma of the mouse prostate (TRAMP) and LPB-Tag transgenic mice (LADY). The most well characterized and used transgenic prostate cancer model is TRAMP mice [72,73]. In these mice the expression of the SV40 large and small T antigen is driven by a region of the prostate-specific rat probasin promoter [74,75]. TRAMP mice develop high-grade PIN at 12 weeks old with poorly differentiated and invasive carcinoma appearing between 18-30 weeks and metastasizing to lymph nodes and lungs and occasionally kidney, bone and adrenal glands [76].

The LADY model uses a large fragment of the rat probasin promoter to direct prostate SV40 large T antigen expression. Although tumor formation varies, typically, at 10 weeks, LADY mice develop PIN followed by high grade PIN at 20 weeks followed by poorly or undifferentiated cancer [77,78]. Male transgenic mice that carry the C3(1) SV 40 early region transgene develop PIN and invasive carcinoma after 8 and 28 weeks, respectively. Another mouse model that carries the fetal globin/SV40 early region transgene develops tumors in the ventral and dorsolateral prostate. Seventy-five percent of heterozygous and 100% for mice homozygous for the transgene develop tumors. The development of tumor in this model is 75% for mice. Mice with the Probasin/rasT24 transgene develop hyperplasia in both the ventral and dorsolateral prostate; however, PIN is not seen in this model [71].

Cell Culture Studies with Finasteride and Dutasteride

Finasteride (1-5 μM) significantly reduced the growth of LNCaP cells treated with testosterone or dihydrotestosterone by as much as 50-60% [79,80]. Dutasteride (0.5-10 μM) significantly inhibited >99% of the conversion of ^{3}H testosterone to ^{3}H-DHT in LNCaP cells [81]. Dutasteride (1 μM) reduced both viability and cell numbers while 10 μM reduced cell proliferation by approximately 50% in LNCaP cells. [3]. Finasteride (0.4-1.6 μM) significantly increased the proliferation of the normal prostate cell line CRL-2221, DU-145, PC-3 and LNCaP cells. Increasing finasteride doses beyond 1.6 μM had no effect on the growth of CRL-2221s, LNCaPs and PC-3s, but significantly decreased growth of DU-145 cells. Dutasteride (0.4-50 μg/well) significantly inhibited the growth of PC-3, DU-145, CRL-2221 and LNCaP cells [82]. Finasteride (0-50 μM) significantly reduced cell viability in LNCaP, PC3 and RWPE-1 cells. Dutasteride (0-50 μM) significantly inhibited the growth of RWPE-1, LNCaP and PC3 cells [83].

Animal Studies with Finasteride and Dutasteride

Finasteride (5 and 20 mg/kg/day) administered to young adult male Sprague-Dawley rats for 28 days significantly reduced ventral prostate, seminal vesicle, and epididymis weights [53]. In a similar study, finasteride (25 mg/kg/day) administered to rats for 7 days significantly reduced ventral prostate weight and decreased 5α-reductase activity [55]. Administration of a higher dose of finasteride (160 mg/kg/day) for 15 days to rats significantly reduced dorsolateral prostate DHT, ventral prostate DHT and circulating DHT levels by 86%, 94% and approximately 98%, respectively. These reductions in prostatic DHT levels were also associated with significant decreases in dorsolateral and ventral weights by 39% and 46%, respectively. In a different rat

study, finasteride treatment (80 mg/kg/day) for 6 months significantly decreased dorsolateral and ventral lobe weights by 46% and 67%, respectively [56].

Dutasteride (2 mg/kg every 2 days) treatment for 4 weeks of weeks significantly decreased LADY mouse ventral prostate, dorsolateral prostate and seminal vesicle weights. A lower dose of dutasteride (0.2 mg/kg every 2 days) also significantly decreased seminal vesicle and dorsolateral prostate weights, but not ventral prostate weights. When treatment initiation was either delayed for 4 weeks or treatment extended to 8 weeks, dutasteride (1 mg/kg per day) significantly reduced dorsolateral prostate and seminal vesicle weights, but not ventral prostate weights [54].

Finasteride (70 mg/kg/day) and dutasteride (100 mg/kg/day) treatments significantly decreased LNCaP prostate cancer growth although the latter treatment was better at equimolar concentrations. Finasteride treatment (0.7, 7, 70 mg/kg/day) in rats bearing the Dunning R-3327H rat prostate tumors reduced the weight and DHT concentration of ventral prostate, but had no effect on tumor weights. However dutasteride (1, 10, 100 mg/kg/day) significantly reduced ventral prostate weight, DHT concentrations and Dunning R-3327H tumor growth [19]. These Dunning R-3327H finasteride results are similar to another study that found a significant reduction in prostate and seminal vesicles weights after finasteride (5 mg/kg body weight/6 day/week) treatment, but no effect on Dunning R3327-H tumor area or weight [20].

Clinical Trials with Finasteride and Dutasteride

The Prostate Cancer Prevention Trial (PCPT), a randomized, double-blind, placebo-controlled clinical trial of 18,882 men with normal digital rectal examination and PSA $\leq$ 3 ng/ml tested the potential of finasteride to prevent prostate cancer in men aged $\geq$55 years old. Men were randomly assigned to finasteride (5 mg/day) or placebo and after seven years finasteride

significantly reduced the prevalence of prostate cancer by 24.8%, although significantly higher Gleason scores were also observed in the finasteride group [17]. Similarly, the Reduction by Dutasteride of Prostate Cancer Events (REDUCE) was a placebo-controlled, randomized, double-blind clinical trial that recruited 8,000 men ≥50 years old with one negative prostate biopsy and a prostate specific antigen of between 2.5-10.3 ng/ml. Men were randomly assigned to receive a daily dose of 0.5 mg dutasteride or placebo and after 4 years there was a significant 23% reduction in prostate cancer incidence in the dutasteride group [18]. In another trial, 46 men with clinical stage T1 and T2 prostate cancer were randomly assigned to receive 5mg of dutasteride or placebo daily for 6-10 weeks before radical prostatectomy. Dutasteride significantly decreased intraprostatic and serum DHT levels [84]. Another 4-month randomized study of 81 men who received dutasteride at doses of 0.5 or 3.5 mg/day prior to radical prostatectomy found PSA concentrations to be significantly lower in men receiving both doses of dutasteride compared to surgery alone [85].

Chapter 3 - Materials and Methods

Cell Lines

RWPE-1 prostate epithelial cells and WPE1-NA22 cancer cells (ATCC, Manassas, VA) were cultured in serum-free keratinocyte media containing bovine pituitary extract and epidermal growth factor (GIBCO Invitrogen, Carlsband, CA). For the animal study, WPE1-NA22 cells were cultured in 75 cm^2 flasks (Fisher Scientific, Pittsburg, PA), removed with trypsin (Sigma-Aldrich, St. Louis, MO), and centrifuged for 7 minutes with 130 x G at 37°C. Supernatant was removed and cells reconstituted in Matrigel™ (BD Biosciences, Franklin Lakes, NJ) at a concentration of 5000 cells/µL. Twenty µL of Matrigel™ containing ~1 x 10^5 WPE1-NA22 cancer cells was injected into each rear flank of nude mice using a Hamilton syringe holder (Hamilton, Reno, NV) fitted with a 1 mL syringe and a 25 gauge 5/8" needle (both from BD Biosciences).

Animals, Study Diets and Design

Two cohorts of thirty (60 total) 8-week old male, nude mice (Charles Rivers, Wilmington, MA) were individually housed in sterile conditions. Mice were monitored daily, weighed weekly, and provided diets & water *ad libitum*. The Institutional Animal Care and Use Committee (IACUC) of Kansas State University approved this study.

AIN93-G diets (Research Diets, New Brunswick, NJ) contained dutasteride (provided by GlaxoSmithKline Pharmaceuticals, Research Triangle Park, NC) and finasteride (Kemprotec, Middlesbrough, UK) at 83.3 mg/kg of diet to provide ~10mg drug/kg body weight. After receipt, mice were acclimated for 1 week, and then randomized into Control, Pre-Finasteride, Post-Finasteride, Pre-Dutasteride and Post-Dutasteride groups (n =12, Figure 3.1). Pre-groups and post-groups began their treatment diets 1-2 weeks prior or 3 weeks after WPE1-NA22 cell

injection, respectively. The study was terminated at 22 weeks post tumor implantation. Mice were euthanized by CO_2 inhalation and blood was immediately drawn via cardiac puncture, and centrifuged for 1 minute at 2000 x G to obtain serum. Tissues were dissected, flash frozen in liquid nitrogen, and stored in a freezer at -80°C. Tumor area was calculated using the formula: (length of tumor (L)/2 x width (W) of tumor/2) x pi. The average tumor area (Figure 4.2) in a group was calculated by dividing the sum of tumor area for the group by the total number of tumor sites in that group. For mice with no tumors, this was indicated with a zero. For tumor area in Figure A.1, tumor area was calculated by dividing the sum of tumor area for the group by the number of sites with tumors in that group.
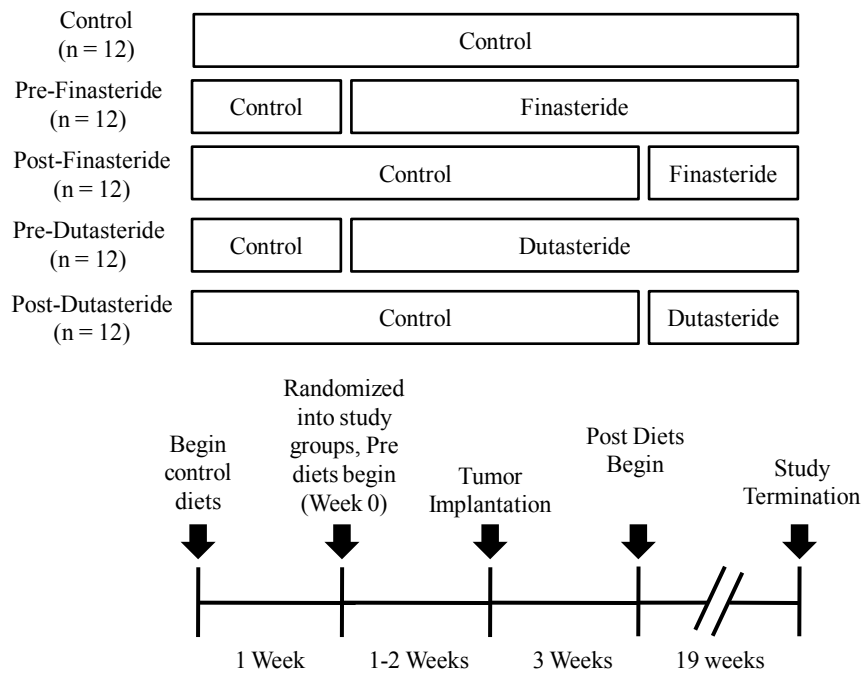

Figure 3.1 Study design- After receipt, mice were acclimated for 1 week, and then randomized into Control, Pre-Finasteride, Post-Finasteride, Pre-Dutasteride and Post-Dutasteride groups (n =12). Pre-groups and post-groups began their treatment diets 1-2 weeks prior or 3 weeks after WPE1-NA22 cell injection, respectively. The study was terminated at 22 weeks post tumor implantation.

In Vitro Androgen Treatment and Cell Viability

RWPE-1 and WPE1-NA22 cells were plated at 10,000 cells per well in 96-well plates (Fisher Scientific, Pittsburg, PA). Twenty-four hours after plating, both cell lines were treated with various doses of testosterone (0.1 nM-30 nM), dihydrotestosterone (0.03 nM-100 nM), (both from Sigma Aldrich, St. Louis, MO) and the synthetic androgen mibolerone (0.01 nM-20 nM) (PerkinElmer, Waltham, MA) in 0.1% ethanol. Media and treatments were changed every 24 hours during the 5 day treatment period. Cell viability was determined using the CellTiter 96 AQueous One Solution Assay (Promega Corporation, Madison, WI) with a Bio-Tek uQuant Plate reader (BioTek, Winooski, VT).

Statistical Analysis

Data was analyzed using SAS 9.2 (SAS Institute Inc., North Carolina) with $p<0.05$ considered statistically significant. ANCOVA with cohort as the covariate was used to initially analyze the animal study results. The covariate did not account for a significant amount of variance in all analyses, thus it was removed and ANOVA with Tukey's test was used on pooled data from the two cohorts. Natural logs were used when data did not meet model assumptions. Kruskal Wallis non-parametric one-way ANOVA was used for tumor incidence. Androgen treatment cell viability data was analyzed using ANOVA with Dunnett's test

Chapter 4 - Results

Final body weights of the Pre-Finasteride group were significantly higher than the control (Table 4.1, $p < 0.05$); despite there being no difference in daily feed intake among the groups, which ranged from 4.70 to 4.87 g/day. Tumor incidence was high, and not significantly different, between groups ranging from 86.4% to 95.5% (Figure 4.1). There was also no significant difference in tumor weights and tumor areas between groups (Table 4.1 & Figure 4.2). This was likely a result of poor tumor growth, as the largest average tumor diameter was 4.33 mm during the 22 week study. However, both finasteride and dutasteride significantly decreased prostate and seminal vesicle weights as a percent of body weight. In addition, there was a significant decrease in seminal vesicle weights in dutasteride groups versus finasteride groups (Table 4.1) The reduction in these androgen-sensitive tissues suggests that finasteride and dutasteride were exerting their anti-androgenic action.

One explanation for this poor growth may be that WPE1-NA22 cells might not be androgen-sensitive like their parent RWPE-1 human prostate epithelial cells have been reported to be [60]. Thus, RWPE-1 human prostate epithelial and WPE1-NA22 cancer cell lines were treated with varying concentrations of the natural androgens testosterone and dihydrotestosterone and the synthetic androgen mibolerone. We found no significant difference in cell numbers in either cell type when treated with varying concentrations of androgens (Figures 4.3 & 4.4).

Table 4.1 Final body weights, tumor incidence, tumor weights, seminal vesicle weights as a percentage of body weight, and prostate weights as percentage of body weights[1].

Groups	Final body weights[2] (g)	Final Tumor Incidence[3] (%)	Tumor weights[3] (mg)	Seminal vesicle weights[2] (% body weight)	Prostate weights[2] (% body weight)
Control	30.6 ± 0.6^a	87.5	35 ± 7	0.92 ± 0.05^a	0.42 ± 0.05^a
Pre-Finasteride	33.2 ± 0.7^b	86.4	25 ± 3	0.34 ± 0.02^b	0.23 ± 0.02^b
Post-Finasteride	30.9 ± 0.8^a	95.5	30 ± 4	0.38 ± 0.03^b	0.27 ± 0.02^b
Pre-Dutasteride	29.4 ± 0.9^a	95.0	36 ± 8	0.21 ± 0.01^c	0.26 ± 0.02^b
Post-Dutasteride	30.2 ± 0.6^a	86.4	22 ± 2	0.23 ± 0.02^c	0.23 ± 0.03^b

[1] Data are means ± SEM, values with different letters are statistically different

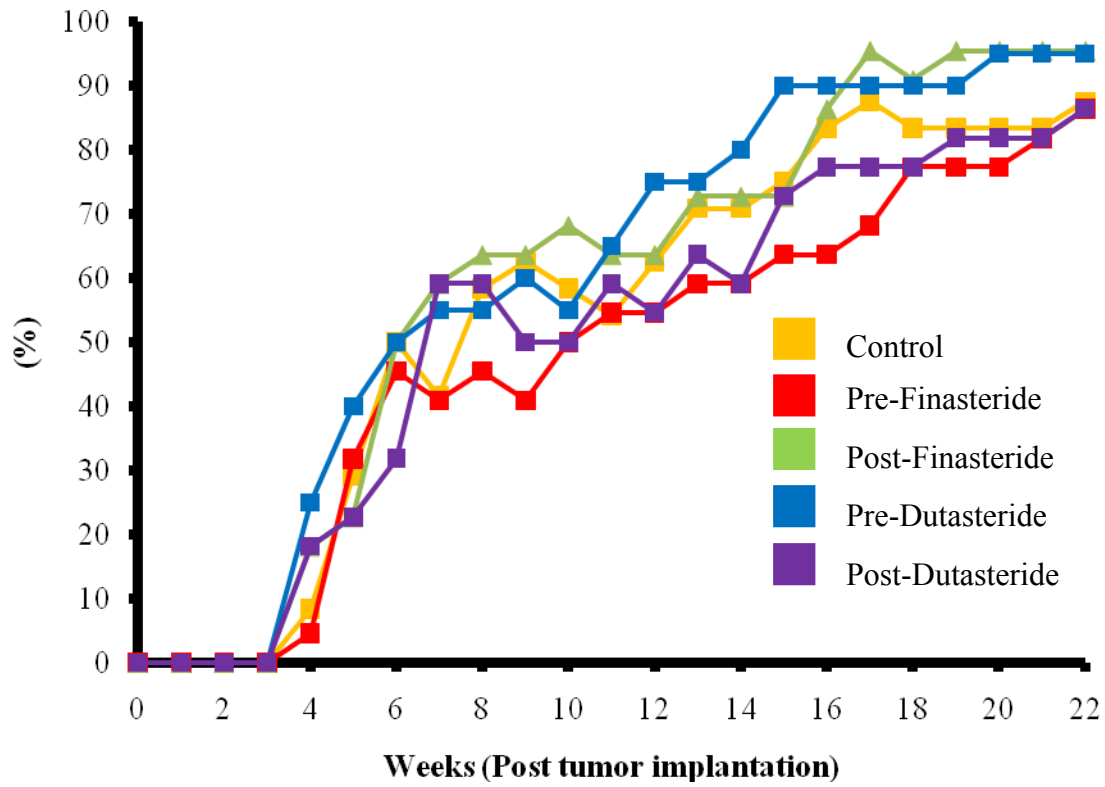[2] n = 10-12

[3] n = 20-24

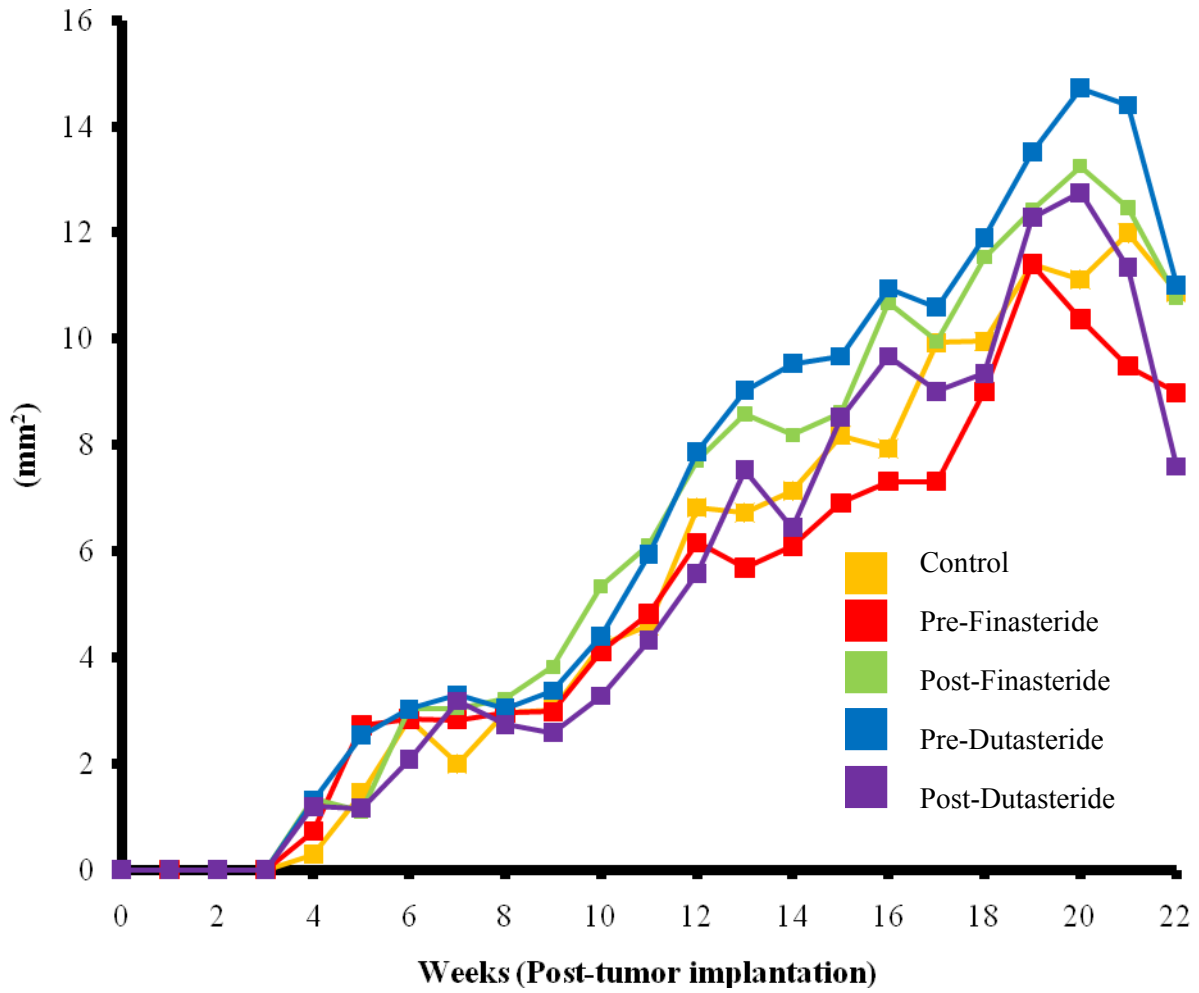Figure 4.1 Tumor incidence (n = 20-24), no significant differences

Figure 4.2 Tumor area (n= 20-24), no significant differences

Figure 4.3 RPWE-1 (plated at 10,000 cells/well) cell viability is not altered in response to daily treatment of testosterone (100 pmol - 30,000 pmol), dihydrotestosterone (30 pmol - 100,000 pmol) and mibolerone (10 pmol - 20,000 pmol) after a 5-day treatment period.
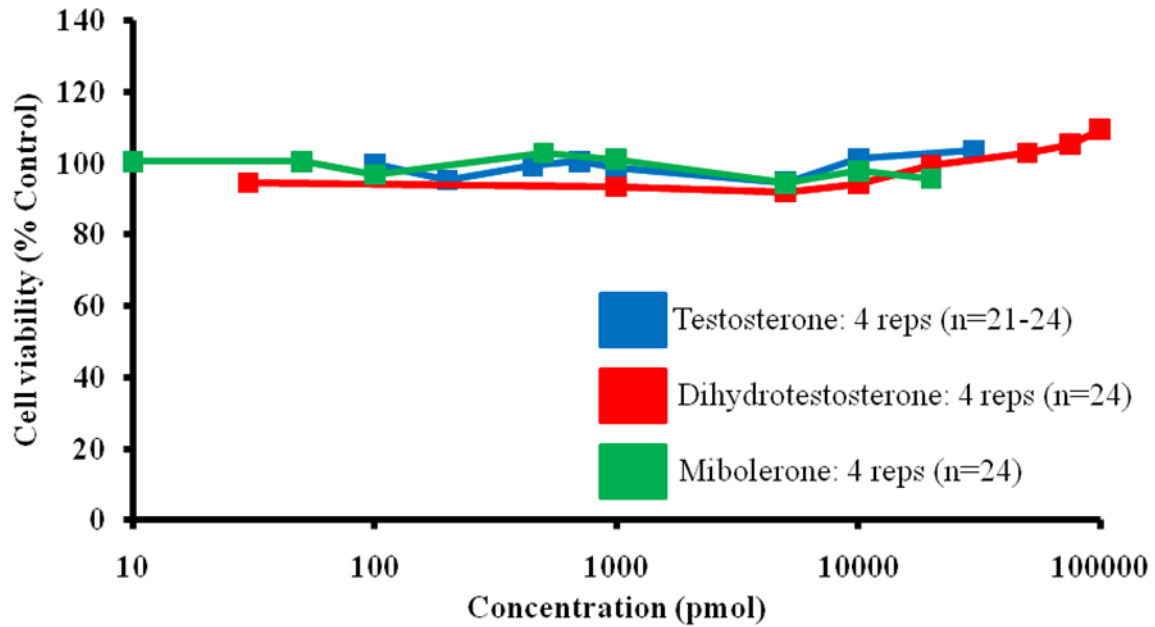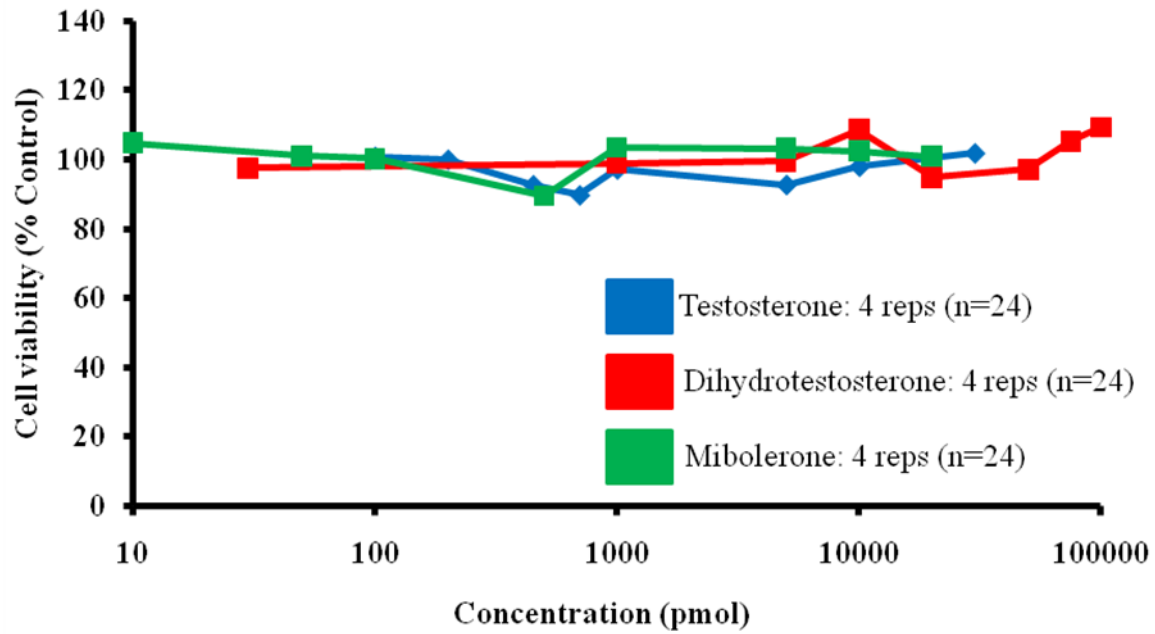
Figure 4.4 WPE1-NA22 (plated at 10,000 cells/well) cell viability is not altered in response to daily treatment of testosterone (100 pmol - 30,000 pmol), DHT (30 pmol - 100,000 pmol) and mibolerone (10 pmol - 20,000pmol) after a 5-day treatment period.

Chapter 5 - Discussion

In this study, we examined the effects of two 5α-reductase inhibitors, finasteride and dutasteride, pre and post tumor injection on the growth of WPE1-NA22 xenografts in nude mice. Tumor incidence was high for all groups ranging from 86.4% to 95.5%, which is similar to the tumor incidence reported in previous Dunning R-3327H rat prostate cancer studies [20,86]. However we were surprised by the poor growth of WPE1-NA22 xenografts in nude mice. The average tumor diameter from largest group was 4.33 mm, which is small compared to the ~7.26 mm diameter that we back calculated from tumor volume previously reported for WPE1-NA22 xenografts seven weeks after tumor implantation [22].

One factor that could have potentially influenced this variation in tumor size between both studies is the difference in number of WPE1-NA22 cancer cells injected into the flanks of mice. Webber and colleagues subcutaneously injected 5×10^5 WPE1-NA22 cells which is five times more cells than what we injected in our study [22]. We injected fewer cancer cells because we were concerned that the tumor growth would be too rapid, given the reported size reported at 7 weeks compared to Dunning R3327H tumors that are not even palpable until 9-10 weeks after tumor implantation.

Another possible explanation for the poor growth of WPE1-NA22 xenografts in nude mice could be due to differences in the source of nude mice for our study and that used by Webber and colleagues. Additionally it is possible that WPE1-NA22 cells are not androgen-sensitive, and thus do not respond to androgens for growth. We set out to investigate the latter possibility by determining whether WPE1-NA22 cells are androgen-sensitive like their parent cell line RWPE-1 human prostate epithelial cells [60].

Growth of RWPE-1 cells increased in a dose-dependent manner when treated with mibolerone (0.01-10 nM) [60]. We followed the methodology of Bello and colleagues who plated RWPE-1 cells at a density of 10,000 cells/well in 96-well plates and treated them with mibolerone (0.01-10 nM) for 5 days to see if we could repeat their results. The only changes were that our highest mibolerone treatment (20 nM) was double their highest mibolerone treatment (10 nM), and we also treated with testosterone and DHT. We chose concentrations of testosterone and DHT based on what several literature reported on cell growth of RWPE1 and WPE1-NA22 cells to the three different androgens [60,79,81]. It is important to note that physiological levels of both intraprostatic testosterone (~0.2-0.7 nM) [53] and dihydrotestosterone (5-18 nM) [19,87,88] in humans fell within the range of concentrations used. Interestingly, we found no difference in cell viability in either cell line in response to various concentrations androgen treatments. Thus, our results do not support that RWPE-1 cells are androgen-sensitive as they have been reported to be previously [60]. The only difference in methodology between the studies was the cell viability assays used. Bello et al. used methylene blue while we used MTS. Nevertheless, it is unlikely that these methodology differences explain the difference in outcomes.

The lack of androgen-sensitivity exhibited by WPE1-NA22 cells may explain the poor tumor growth observed. Furthermore, a recent publication found androgen receptor and 5α-reductase 1 protein levels to be undetectable in the nucleus and cytoplasm, respectively of RWPE-1 cells [83]. It is possible that the cell lines derived from this parent cell line may also have similar levels of these key proteins. Taken together, these results will have to be considered before using or interpreting results in RWPE-1 and its carcinogen-derived cell lines.

Another surprising result was significantly higher body weights in the Pre-Finasteride group despite no alteration in food intake. We believe based on the trend in growth seen in the Pre-Finasteride group that, that group may have been heavier from randomization because they were the heaviest group after only 1 week on the diet and continued to be heavier throughout the study. Data supporting finasteride increasing weight gain is sparse with a trial using finasteride reporting annual weight gain in men with high grade and low grade prostate cancer to be 0.01% and 0.25% higher than placebo respectively [89]. Similarly a study to determine the long term effect of finasteride on rats, found no significant change in body weight with finasteride treatment [90]. Diets in all treatment groups seemed however to have been well tolerated and there were no noted adverse effects.

Despite no effect on tumor growth, all the treatments significantly decreased prostate and seminal vesicles weights. Finasteride reduced prostate weight in Pre and Post finasteride groups by 59% and 64.6%, respectively, while dutasteride decreased prostate weight in Pre and Post dutasteride groups to 62% and 56%, respectively. In rats finasteride (5, 20 and 25 mg/kg body weight) caused a reduction in the prostate to 49%, 54%, and 55% of control, respectively [91,92]. Canene-Adams et al. also reported that finasteride (5 mg/kg/body weight) significantly reduced rat prostate and seminal vesicle weight [20]. In our study, there was a significant decrease in seminal vesicle weights in dutasteride groups versus finasteride groups. Finasteride reduced seminal vesicles in Pre and Post-finasteride groups to 36% and 42% of control, respectively, while dutasteride decreased seminal vesicle weights to 23% and 25% of control, respectively. Mice in our study received approximately 13 mg/kg body weight/day of finasteride and dutasteride which is greater than previous studies that have provided 5 mg/kg body weight and [20] doses of finasteride (0.7, 7 and 70 mg/kg/day) and dutasteride (1, 10, 100 mg/kg/day)

[19]. In summary, although we did not see an effect of either finasteride or dutasteride on the growth of WPE1-NA22 xenografts, the decrease in the prostate and seminal vesicle weights suggest that the inhibitors were effective in inhibiting their respective 5α-reductase enzyme(s).

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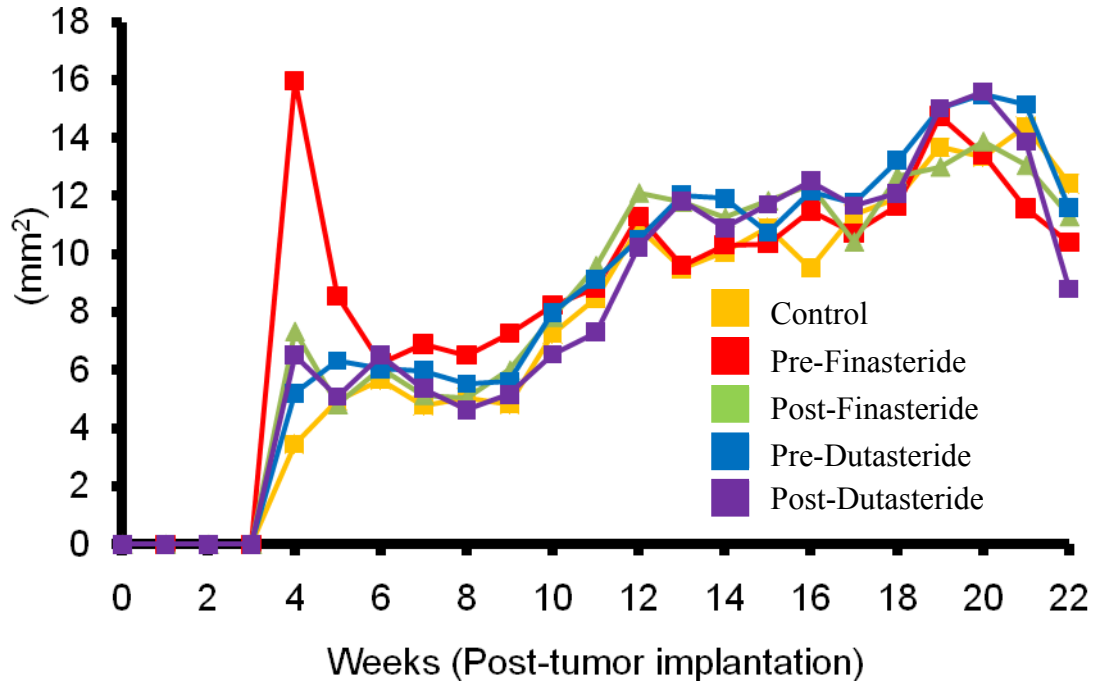# Appendix A - Tumor Area (n = 20-24)

Figure A.1 Tumor area (n= 20-24)
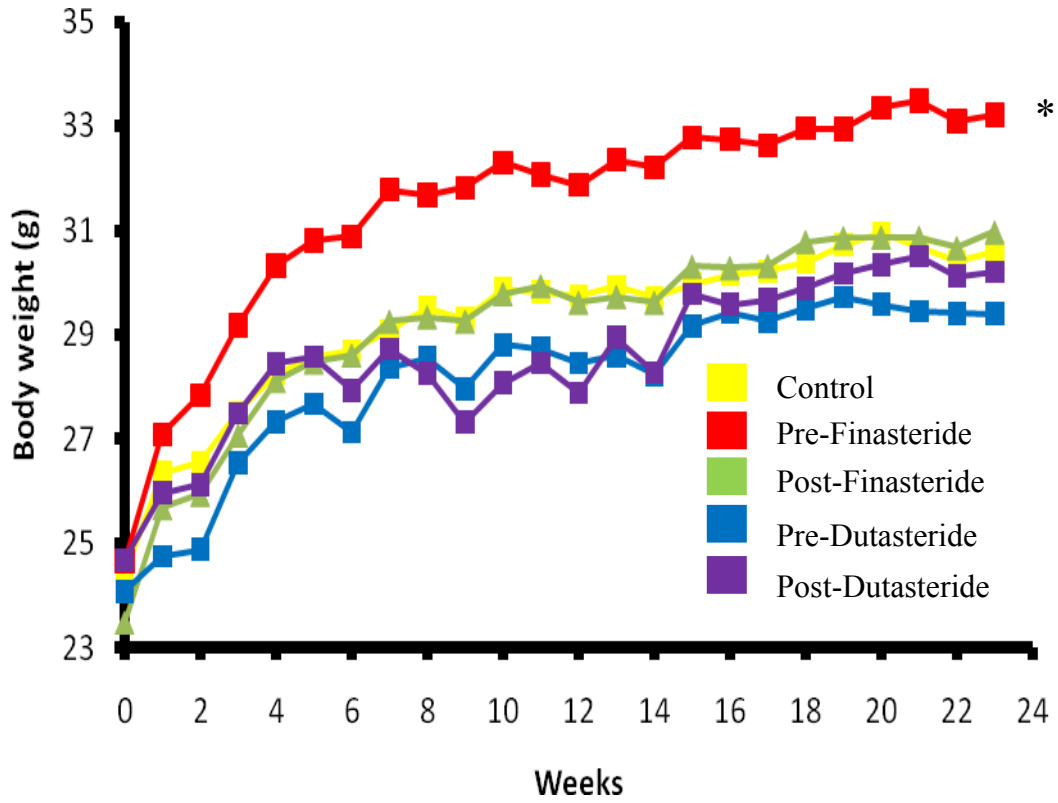
Appendix B - Body Weight (n = 10-12)

Figure B.1 Body weight (n = 10-12)